BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOTRIMAZOLE IN HUMAN PLASMA BY RP-HPLC METHOD

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai.

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

APRIL-2014

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPATTI ROAD,

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Submitted by

(Reg. No.261230752)

Under the Guidance of

Mrs. N.Tamilselvi, M.Pharm., Asst.Professor

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CERTIFICATE

This is to certify that, the work embodied in the thesis entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOTRIMAZOLE IN HUMAN PLASMA BY RP-HPLC METHOD"*** *is a bonafide research work carried out by* ***Reg. No: 261230752****, Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu under the guidance of* ***Mrs. N.Tamilselvi****,* ***Asst professor,*** *Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy during the academic year 2013-2014.*

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Place: COIMBATORE **Dr. A. Rajasekaran, M. Pharm, Ph.D.,**
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I am fully satisfied with her performance and work with great pleasure. I forward this Dissertation work for evaluation.

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Place: Coimbatore. **Mrs. N.Tamil Selvi M.Pharm.,**

Asst.Professor.

DECLARATION

I am here by stating that, to the best of my knowledge and belief, the project report entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOTRIMAZOLE IN HUMAN PLASMA BY RP-HPLC METHOD"*** *being submitted for the partial fulfillment of Master of Pharmacy in Pharmaceutical Analysis for the academic year 2013-2014 of KMCH College of Pharmacy affiliated to The Tamilnadu Dr. M.G.R. Medical University carried out under the guidance of* ***Mrs. N.Tamilselvi, M.Pharm, Asst.professor****, at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore.*

I abide that all the data presented in this report will be treated with almost confidentiality.

Date: Signature,

Place: Coimbatore. ***Reg No 261230752***

EVALUATION CERTIFICATE

This is to certify that, the work embodied in the thesis entitled ***"BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOTRIMAZOLE IN HUMAN PLASMA BY RP-HPLC METHOD"*** *submitted by* ***Reg. No: 261230752,*** *to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of* ***Master of Pharmacy****, in* ***Pharmaceutical Analysis****, is a bonafide research work carried out by the candidate at K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, the same was evaluated by us during academic year 2013-2014.*

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

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Above all I dedicate myself before the unfailing presence of ***GOD*** *and constant love and encouragement given to me by my beloved* ***Father, Mother, Brothers, Sisters*** *and all of my family members who deserves the credit of success in whatever work I did.*

Reg No 261230752

ABBREVATIONS

HPLC	High Performance Liquid Chromatography
UV	Ultra violet
BA	Bioavailability
MW.	Molecular weight
e.g.	Example
i.e.	That is
%	Percentage
PDA	Photo Diode Array
IS	Internal Standard
ACN	Acetonitrile
MET	Methanol
RF	Response Factor
Mg	Milligram
mL	Milliliter
mM	Milli molar
µg	Microgram
w/w	Weight by weight
v/v	Volume by volume
µg/ml	Microgram per milliliter
ng /ml	Nanogram per milliliter
pH	Hydrogen ion concentration
^{0}C	Degree centigrade

T	Time
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
Tab.	Table
M.P.	Melting Point
AUC	Area under curve
CV	Coefficient of variance
RSD	Relative standard deviation
L/h	Liter per hour
L/kg	Liter per kilogram
Rpm	Revolution per minute
Rt	Retention time
LLE	Liquid liquid Phase Extraction

INDEX

1. INTRODUCTION

Pharmaceutical Analysis[1-2] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air.

TYPES

There are mainly two types of chemical analysis:

1. Qualitative (Identification)
2. Quantitative (Estimation)

1. **Qualitative analysis** is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, color change reactions, melting point and boiling point test etc.

2. **Quantitative analytical** techniques are mainly used to quantify any compound or substance in the sample. These techniques are based on (a) the quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained. (b) The characteristic movement of a substance through a defined medium under controlled conditions. (c) Electrical measurement. (d) Measurement of some spectroscopic properties of the compound.

ROLE OF BIOANALYSIS IN PHARMACEUTICAL DRUG DEVELOPMENT [6]

Quantitative determination of drugs and their metabolites in biological fluids is termed as bioanalysis. This technique is used very early in the drug development process to provide support to drug discovery programs on the metabolic fate and pharmacokinetics of chemicals in living cells and in humans.

Bioanalytical methods play a major role in estimating the drugs, interferences, metabolites from various matrices such as pure drug, dosage form, intermediates and biological fluids. Drug assay technology is now sufficiently advanced for it to be possible to measure the plasma concentration of majority of drugs used in clinical practices. They are useful to measure plasma concentration of drugs to confirm adequate dosage, to identify signs of possible drug toxicity, the response of patients to drug therapy and drug interactions.

When a drug is administered orally it passes through the GIT and enters the systemic circulation undergoes metabolism, finally it is excreted as such or in the form of its metabolites. The studies on biological fluids is very challenging and time consuming , but these studies are necessary and utmost important because biological fluids like blood, urine, cerebrospinal fluid and milk etc containing a relative quantity of drug and their metabolites can be known. Bio-analytical method which gives accurate and reproducible results has been increased significantly now a day.

Therapeutic efficacy of the particular drug can be known by bioanalysis. In Pharma field bioanalysis plays a significant role .Bioanalysis involves the following steps.

- Selection and collection of biological fluid.
- Preparation of sample –Analyte extraction from biological matrix.
- Analyte detection done by various methods.

In bioanalytical drug analysis, common matrices are venous blood and plasma. Venous blood, with anticoagulant, is after centrifugation separated into plasma and blood cells. Centrifuged blood consists of 55% plasma and 45% blood cells. The plasma matrix is composed of some 90% water with remaining 10% being mostly plasma proteins. Sampling of biological matrix is the first step in the

analytical chain, and these samples must regularly be stored in a cooler or freezer before assay. When the samples have arrived at the laboratory, they need to be prepared for analysis. The aim is to improve the assay by removing interferences in the matrix, and often also concentrating the analyte. The need of sample preparation is determined by the complexity of the samples and by the application of the analytical method.

Detection of drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involved i.e. solvent extraction and chromatography are employed to effectively separate drug components form endogenous biological materials. The sensitivity and selectivity of the assay method was limited by the efficiency of the clean up methodology [(7)].

If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Thus, plasma generally is preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for bioanalytical studies and may require protein denaturation steps before further processes.

If plasma or serum is used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 5000 rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as pasture pipette, to a clean container of appropriate size of storage.

DIFFERENT TERMINOLOGY USED IN BIOANALYTICAL DEPARTMENT

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness.

Analyte: A specified chemical moiety being measured or which can be intact drug, biomolecule or its derivative, metabolite and/or degradation product in a biological matrix.

Analytical run (or batch): A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or

batches) may be completed in one day, or one run (or batch) may take several days to complete.

Biological matrix: A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum and various discrete tissues.

Calibration Standard: A biological matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

Internal standard: Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Matrix effect: The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A comprehensive description of all procedures used in sample analysis

Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Processed: The final extract (prior to instrumental analysis) of the sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reproducibility: The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

Sample: A generic term encompassing controls, blanks, unknowns and processed samples as described below.

Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Quality control sample (QC): A spiked sample used to monitor the performance of a Bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Unknown: A biological sample that is the subject of the analysis.

Selectivity: The ability of the Bioanalytical method to measure and differentiate the analytes in the presence of the components that may be expected to be present. These could include metabolites, Impurities, degradants or matrix components.

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

VALIDATION:

Full validation: Establishment of all validation parameters to apply to sample analysis for the Bioanalytical method for each analyte.

Partial validation: Modification of validated bioanalytical methods that do not necessarily call for full validation.

Cross-validation: Comparison validation parameters of two bioanalytical methods.

STORAGE REQUIREMENTS FOR BIOLOGICAL SAMPLES [8]

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride was immediately added after collection helps to prevent decomposition.

When collecting and storing biological samples, the analyte should be contaminated with storage vessels. For example, plastic –ware frequently contains high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger-plugs of vacutainers are known to contain tri-butoxyethyl phosphate, which can be interfering certain drug analysis.

EXTRACTION PROCEDURE FOR BIOLOGICAL SAMPLE

Sample preparation is a technique used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. When samples are biological fluids such as plasma, serum or urine, this technique is described as bioanalytical sample preparation.

Objectives of Bio-analytical sample preparation:-

1. Removal of unwanted matrix components (primarily protein) that would interfere with analyte determination.
2. Concentration of analyte to meet the detection limits of the analytical instrument.
3. Exchange of the solvent or solution in which the analyte resides so that it is compatible with mobile phase for injection into a Chromatographic system.
4. Dilution to reduce solvent strength or avoid solvent incompatibility.
5. Stabilization of analyte to avoid hydrolytic or enzymatic degradation.

Blood, serum and plasma contain approximately 10,000 different proteins with a total concentration of 6–8 g/dL, and urine contains 50–100 mg of protein/dL. Plasma also contains approximately 3 mg/mL of sodium, 0.2 mg/mL of potassium, 15–38.5 mg/dL of urea, and many other organic and inorganic compounds. With such complex mixtures and the dynamic ranges of proteins, fatty acids, lipids and salts, it is clear that it is important to remove these components from the analysis, as these factors could confound the analysis and flood the detector with irrelevant ions.

GENERAL TECHNIQUES FOR SAMPLE PREPARATION [9]:-

Many different sample preparation techniques are available for choosing a method to perform bioanalytical sample preparation. These techniques vary in many regards, such as simplicity, time requirement (in terms of speed and hands-on analyst time), ease of automation, extraction chemistry expertise, concentration factor and selectivity of the final extract.

Typical choices of sample preparation techniques useful in bioanalysis

- Dilution followed by injection
- Solid Phase extraction
- Protein precipitation
- Filtration
- Liquid-liquid extraction
- Restricted access media

Solid phase extraction

Solid phase extraction (SPE) is one of the most common bio-analytical extraction methods in publication literature, a search of journal articles using the science direct search engine for the term "SPE" yielded 151,017 hits, with 4,657 hits for the year 2010 alone. SPE involves a solid-liquid phase separation of the analytes from the biological sample, by the selective transfer between a liquid and solid state [9]. The analyte is physically separated from the biological matrix by the differential interacting with a solid phase sorbent material. These sorbents, packed primarily into either disposable cartridges or discs, can be polar, non-polar or ionic depending on the experimental requirements.

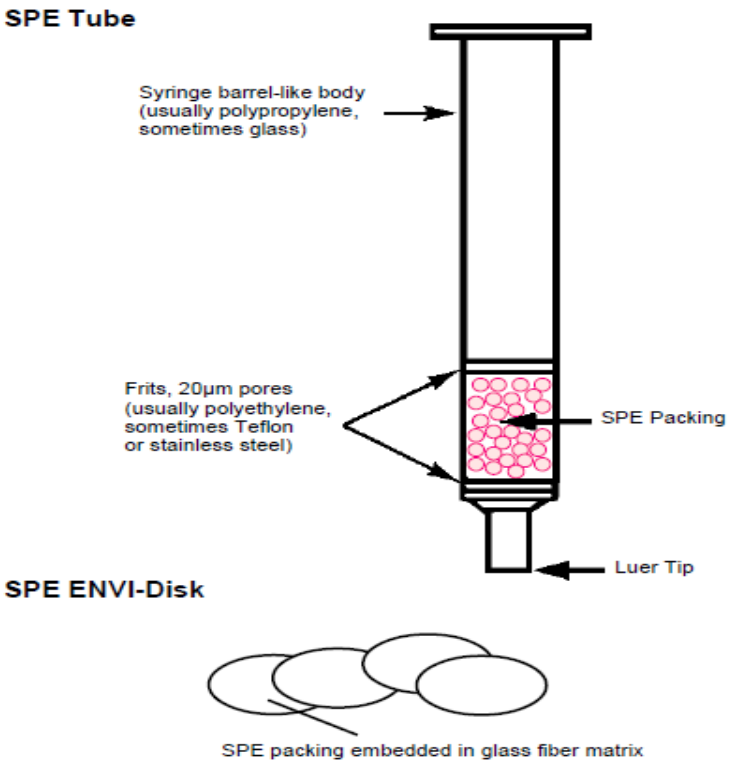


Fig 1: Typical solid phase extraction tube and Disk

Solid phase extraction is based on four distinct protocol steps as illustrated in Fig 2.

Step 1: **Sorbent Conditioning**

Prior to the addition of the sample conditioning and packing of the cartridge is done. In order to remove the impurities a conditioning strong solvent like methanol is passed through cartridge which allows sorbent to be solvated.

Step 2: **Sample Loading**

In a weak solvent the sample is dissolved and passed through the cartridge. Sample is applied with a syringe or pipette or pumped into cartridge .This weak solvent helps in retention of strong analyte.

Step 3: **Washing**

In order, to remove the interferences and impurities the cartridge is washed with water or buffer.

Step 4: **Analyte Elution**

Strong elution solvent is passed through the cartridge and it gives 100% collection of analyte from this method.

The availability of commercially available solid phase extraction material, cartridges or sorbents, has led to uniformity in analysis and makes method replication and transfer from one lab to another more straightforward. SPE is also frequently used in on-line extraction. Automation of sample clean-up is advantageous as it decreases the high level of manual handling and error associated with manual extraction procedures. Though on-line SPE is faster and allows for increased through-put injections and offers high recoveries, extensive method development is required to optimize many experimental variables.

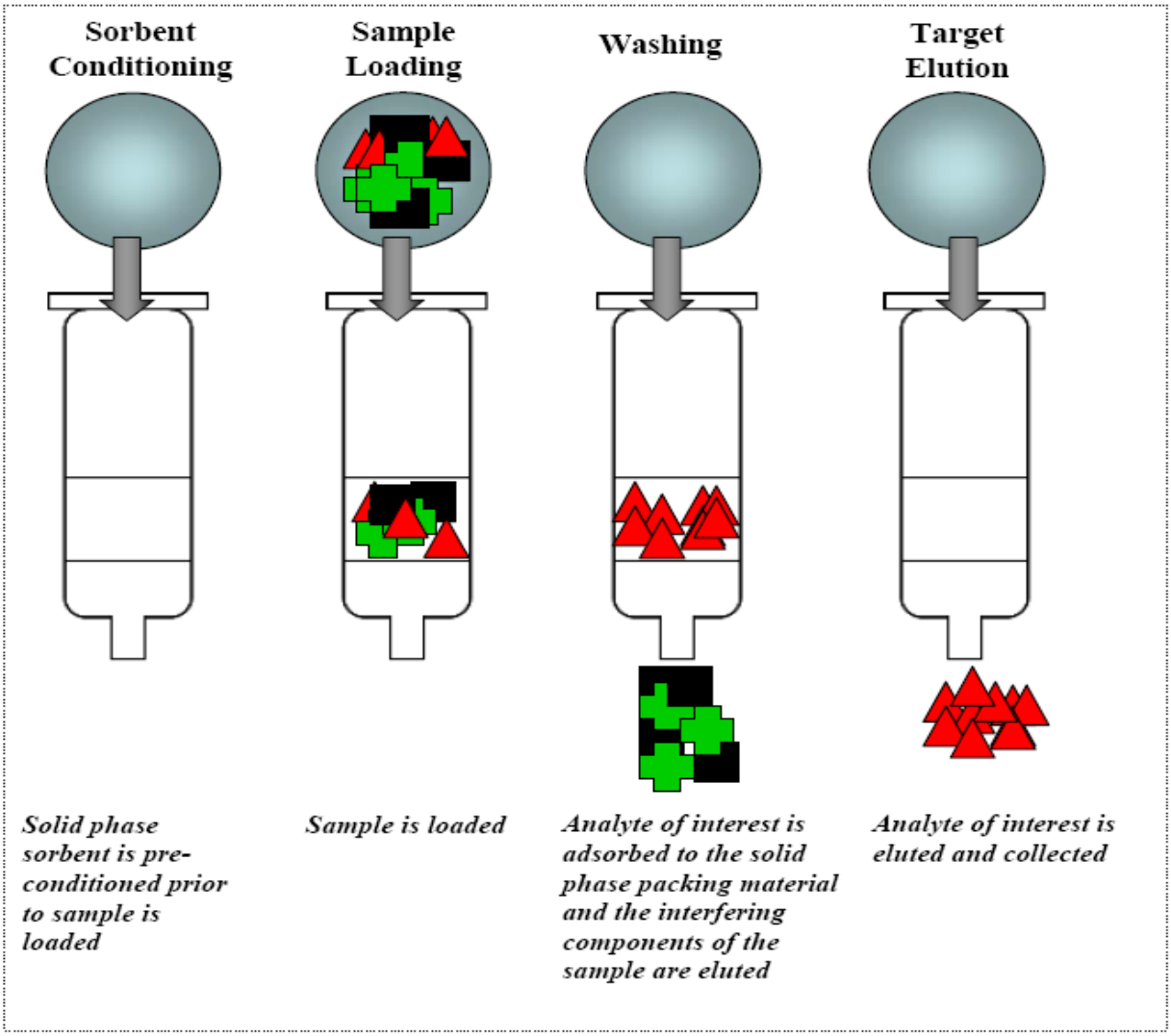


Fig 2: Schematic of solid phase extraction procedure

SOLID PHASE EXTRACTION THEORY

How Compounds Are Retained by the Sorbent

1) Reversed Phase

   (polar liquid phase, nonpolar modified solid phase)

   Hydrophobic interactions

   - nonpolar-nonpolar interactions
   - van der Waals or dispersion forces

2) Normal Phase

   (nonpolar liquid phase, polar modified solid phase)

   Hydrophilic interactions

   - hydrogen bonding

- pi-pi interactions
- dipole-dipole interactions
- dipole-induced dipole interactions

3) Ion Exchange

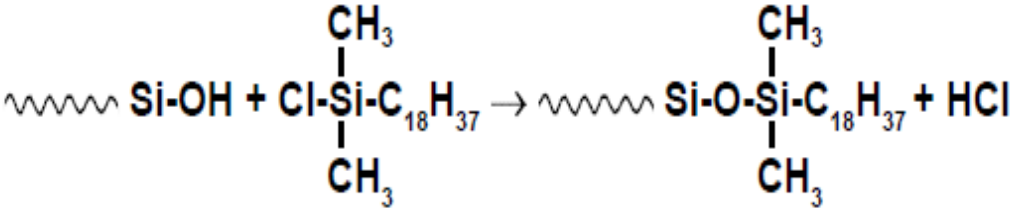
Electrostatic attraction of charged group on compound to a charged group on the sorbent's surface

4) Adsorption

(interactions of compounds with unmodified materials) Hydrophobic and hydrophilic interactions may apply Depends on which solid phase is used

Reversed Phase SPE

Reversed phase separations involve a polar (usually aqueous; see Table An on page 8) or moderately polar sample matrix (mobile phase) and a no polar stationary phase. The analyte of interests typically mid- to no polar. Several SPE materials, such as the alkyl- or aryl-bonded silicas (**LC-18, ENVI-18, LC-8, ENVI-8, LC-4,** and **LC-Ph**) are in the reversed phase category. Here, the hydrophilic silanol groups at the surface of the raw silica packing (typically 60Å pore size, 40µm particle size) have been chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes.



The Role of pH in solid phase extraction

Solutions used in SPE procedures have a very broad pH range. Silica-based packings, such as those used in HPLC columns, usually have a stable pH range of 2 to 7.5. At pH levels above and below this range, the bonded phase can be hydrolyzed and cleaved off the silica surface, or the silica itself can dissolve. In SPE, however, the solutions usually are in contact with the sorbent for short periods of time. The fact that SPE cartridges are disposable, and are meant to be used only once, allows one to use any pH to optimize retention or elution of analytes. If stability of the SPE cartridge at an extreme pH is crucial, polymeric or carbon-based SPE materials such as ENVI-Chrom P or ENVI-Carb may be used. These materials are stable over the pH

range of 1-14.

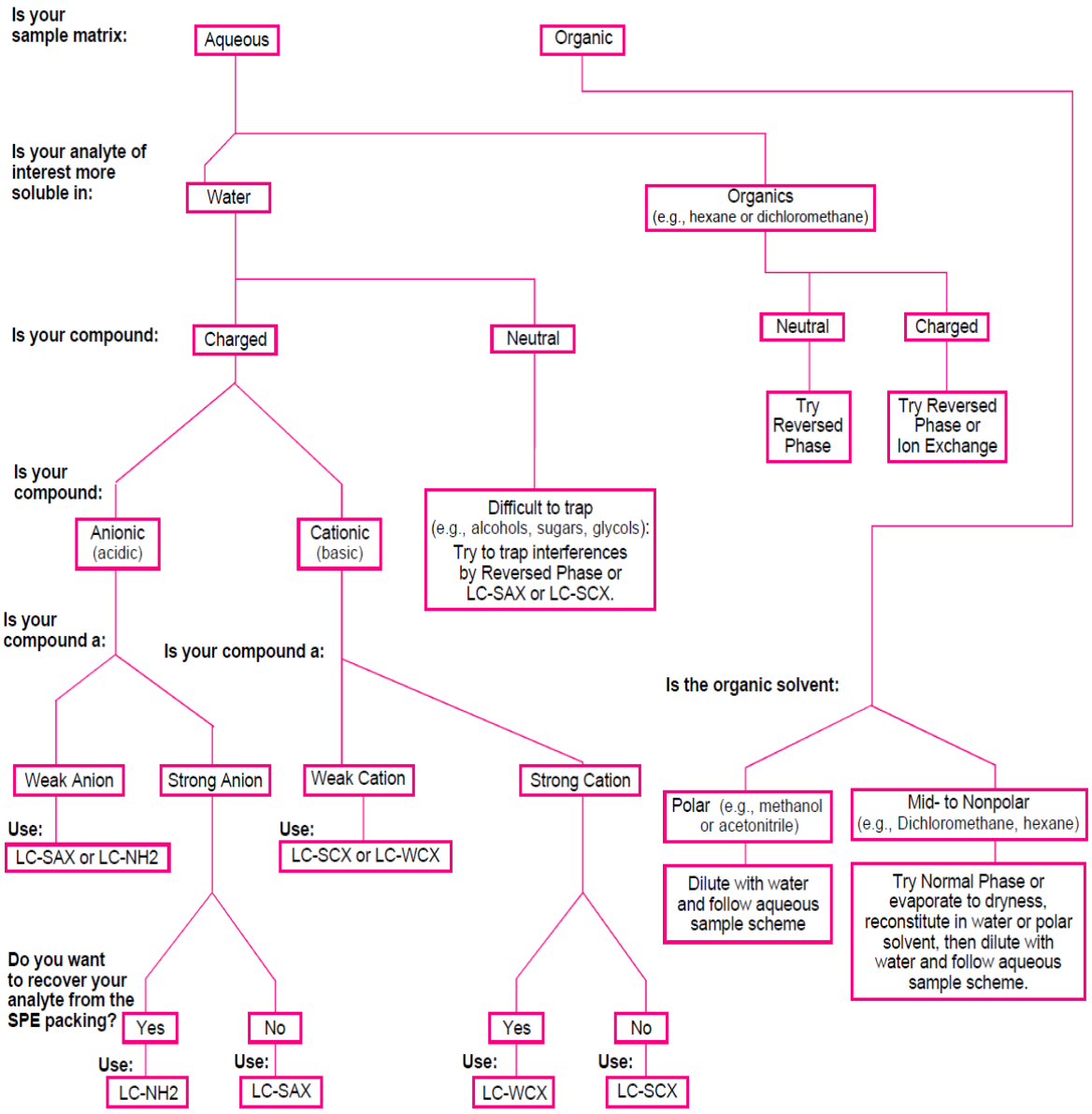
For reversed phase SPE procedures on bonded silicas, if trapping the analyte in the tube is desired, the pH of the conditioning solution and sample (if mostly or entirely aqueous) should be adjusted for optimum analyte retention. If the compound of interest is acidic or basic you should, in most cases, use a pH at which the compound is not charged. Retention of neutral compounds (no acidic or basic functional groups) usually is not affected by pH. Conversely, you can use a pH at which the unwanted compounds in the sample are retained on the SPE packing, but the analyte of interest passes through unretained. Secondary hydrophilic and cation exchange interactions of the analyte can be used for retention at a proper pH. (For more detail, see Secondary Interactions).

For adsorption media (e.g. ENVI-Carb and ENVI-Chrom P) that are used under reversed phase conditions, a pH should be chosen to maximize retention of analytes on the sorbent as with reversed phase bonded silicas. Elution is usually done with an organic solvent, so pH is usually not a factor at this point. Surprisingly, phenols retain better on ENVI-Chrom P when applied in solutions at a neutral pH, where phenols can be charged, than at an acidic pH levels where they are neutral. This shows that adsorption media may have different selectivities than the bonded silicas for certain compounds, and that a range of pH levels of the sample and conditioning solutions should be investigated when using these materials.

Table no 1: Commonly used solvents in solid phase extraction

Polarity			Solvent	Miscible in Water?
Nonpolar	Strong Reversed Phase	Weak Normal Phase	Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Methylene chloride (dichloromethane)	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
			Methanol	Yes
			Water	Yes
Polar	Weak Reversed Phase	Strong Normal Phase	Acetic acid	Yes

Determination of solid phase extraction procedure by sample characteristics



Protein precipitation

Protein precipitation is based on the interaction between the precipitation reagent and protein groups. Soluble proteins generally have a hydrophobic core surrounded by a hydrophilic surface including ionic groups that are not involved in intra-molecular binding. Organic solvents interfere with the intra-molecular hydrophobic interactions of proteins. [10]

The addition of a volume of solvent (frequently acetonitrile) to the serum causes the proteins of the serum to precipitate and leaves the analyte of interest in

the solvent, which can either be injected directly or dried down and reconstituted in a smaller volume to concentration before injection. While this is the fastest and simplest method for sample preparation, it is the most likely to cause ion suppression issues, especially in ESI, where the co-elution of endogenous compounds such as lipids, phospholipids and fatty acids affect the ESI droplet desolvation process. [11]

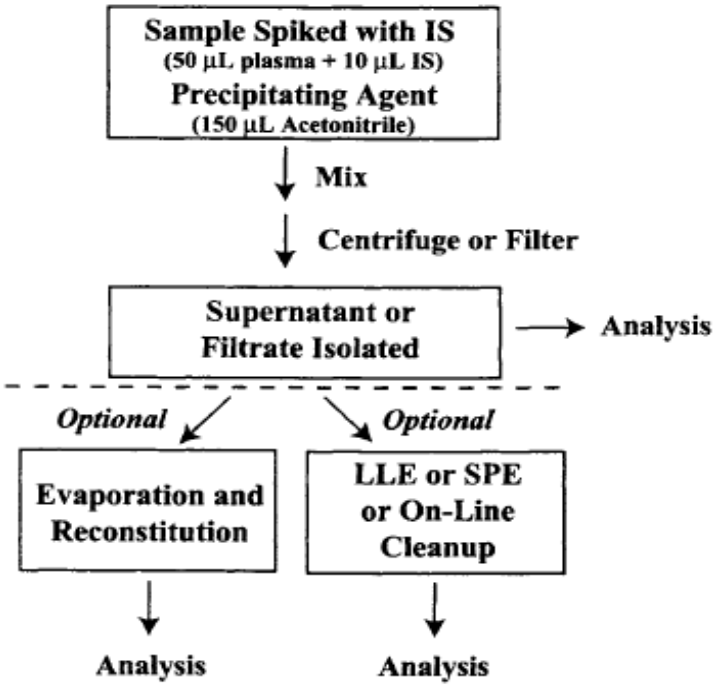


Fig 3: Schematic diagram of Protein precipitation technique.

Liquid-Liquid Extraction [12]

Liquid-liquid extraction (LLE) is the simplest form of the extraction and purification of analytes from liquid samples. The basic principle is the tendency of an analyte to prefer one solvent over another immiscible solvent. The often-quoted partitioning coefficient of a species is Log P. This is defined as the ratio of the concentration in octanol divided by the concentration in water. The Log-P values of a vast number of species have been calculated, and there are computer programs that will calculate Log P and Log D simply from the chemical structure of the species. The use of high-purity solvents can make this technique expensive, especially if large volumes are required and then there is the disposal of these solvents. Carrying out LLE requires the use of two immiscible liquids and soluble samples. It is very useful for separating analytes from interferences by partitioning the sample between these

two immiscible liquids or phases. Typically, with LLE one phase will be aqueous (often the denser or heavier phase) and the second phase is an organic solvent (usually the lighter phase). Hydrophilic compounds prefer the polar aqueous phase, whereas hydrophobic compounds will prefer the organic solvent. Analytes extracted into the organic phase are recovered relatively easily by evaporation of the solvent, whereas analytes extracted into the aqueous phase are often amenable to direct analysis by high-performance liquid chromatography (HPLC). Extraction efficiency can be improved by solvent selection, solvent volume, pH, and by using the salting-out effect.

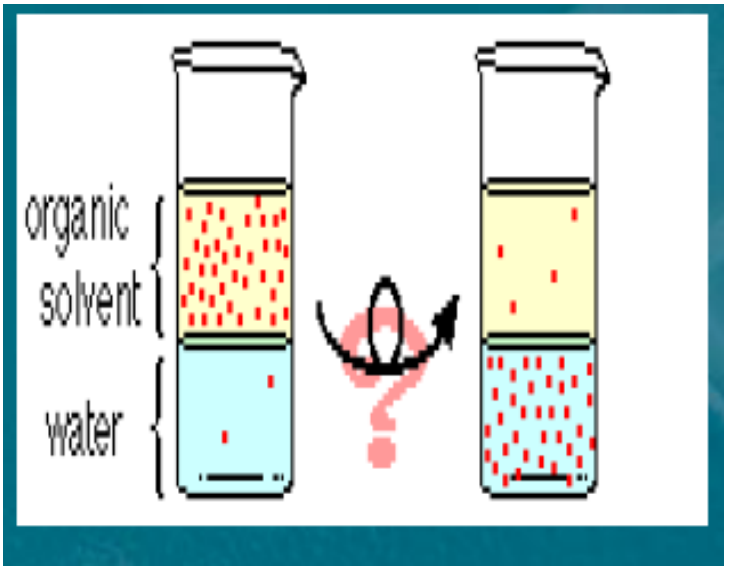


Fig 4: Schematic representation of Liquid – Liquid Extraction

Positive –

- ✓ Known and standard technique.
- ✓ Relatively simple to do as a routine.
- ✓ Trend to micro LLE.
- ✓ Low LOD's are possible.

Negative –

- ✓ Moderate selective (limited in solvent selections).
- ✓ Examine and use pH / ionic strength / temperature to get a selective extraction process.
- ✓ Multiple extractions needed to get the recovery.
- ✓ Often evaporation steps needed.
- ✓ Emulsions formed cause recovery loss.

- ✓ Difficult to automate, semi-automated steps.
- ✓ As routine method labor intensive.

Analytical methods for quantitative determination of drugs in biological fluids:

There are different types of methods for quantitative determination of drugs in biological fluids. According to biological fluid and drug to be quantitated these methods are selected. They are:

a) Methods based on immunoassay procedure
   - ✓ Radioimmunoassay (RIA).
   - ✓ Enzyme-multiplied immunoassay technique.
   - ✓ Enzyme-linked immunosorbent assay (ELISA).

b) Microbiological methods.

c) Capillary electrophoresis.

d) Chromatographic methods.
   - ✓ Gas chromatography (GC).
   - ✓ High performance liquid chromatography (HPLC).
   - ✓ Liquid chromatography-mass spectroscopy (LC-MS).
   - ✓ Gas chromatography –mass spectroscopy (GC-MS).

Chromatographic methods are mostly used and important for the estimation of drugs in biological samples. Chromatography derived from "*chroma"* means "color" and "*graphein"* means "to write". Separation of required analyte from the compounds by using the mobile phase and stationary phase is known as chromatography. Advanced methods for separation of samples from biological fluids are HPLC and their hyphenated methods like LC-MS; GC-MS.

Drugs estimation in biological samples by HPLC

Most of the drugs in biological samples can be analyzed by High performance Liquid Chromatography method because of several advantages like accuracy, precision, rapidity, and specificity, ease of automation and elimination of tedious extraction methods and isolation procedure. Some of the advantages are:

- ➢ Speed (analysis can be completed in 20 min or less)
- ➢ Higher sensitivity (various detectors can be employed)
- ➢ Good resolution (wide variety of stationary phase)
- ➢ Re-usable column (expensive columns but can be used for many Samples)
- ➢ Ideal for the substance of low volatility

- Sample recovery, handling and maintenance were easy
- Instrumentation leads itself to automation and quantization
- Precise and accurate,
- Calculation are done by integrator and
- Suitable for preparative liquid chromatography on a much larger scale.

The different models of separation are in HPLC. Normal phase mode, Reverse phase mode, reversed phase ion pair chromatography, ion exchange chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

HPLC is historically divided into two different sub classes based on the polarity of the mobile and stationary phases.

1. Normal phase high performance liquid chromatography.
2. Reverse phase high performance liquid chromatography.

Normal phase high performance liquid chromatography:

Techniques in which the stationary phase is more polar than the mobile phase is call normal phase high performance liquid chromatography.

✓ Stationary Phase –Polar nature

e.g.: Sio2, Al2O3.

✓ Mobile Phase – Non-polar nature

e.g.: Heptane, hexane, cyclohexane, CHCL3, CH3OH

Mechanism:

- Polar compounds travels slower and eluted slowly due to higher affinity b/w solute and stationary phase.
- No polar compound travels faster and eluted first due to lower affinity b/w solute and stationary phase.
- This technique is not widely used in pharmaceutical separation.

Reverse phase high performance liquid chromatography:

Techniques in which the mobile phase is more polar than the stationary phase is called reverse phase high performance liquid chromatography.

✓ Stationary phase – Non-polar nature.

e.g.: n-octadecyl, n-octyl, ethyl, phenyl diol, hydrophobic polymers.

✓ Mobile Phase – polar nature.

e.g.: Methanol or Acetonitrile/water or buffer sometimes with additives of THF or dioxane.

Mechanism:

- A polar compound travels faster and eluted first due to lesser affinity b/w solute and stationary phase.
- Non polar compounds travel slower and eluted slowly due to higher affinity b/w solute and stationary phase.

Principle:

- ✓ The principle of separation is normal phase mode and reverse phase mode is adsorption. When mixtures of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower.
- ✓ The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.
- ✓ Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

The various component of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or auto), guard column, analytical column, detector, recorder, and /or integrators. Recent models are equipped with computers and software for data acquisition and processing.

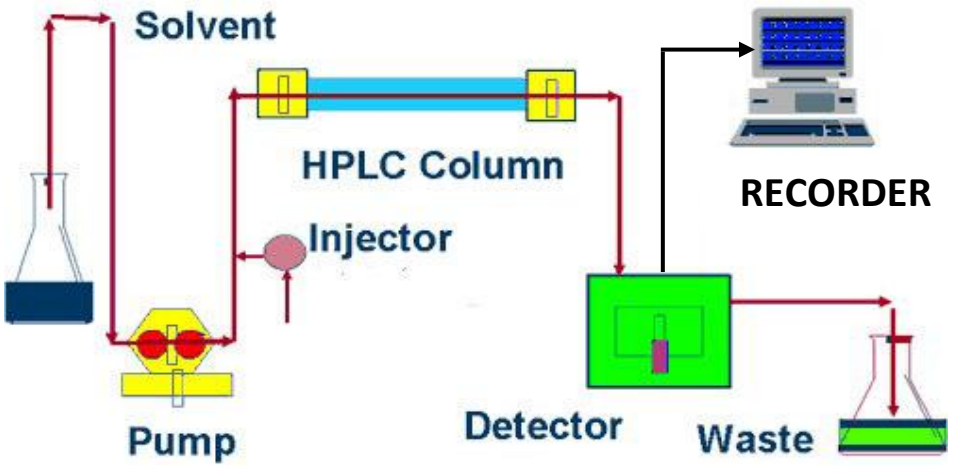


Fig 5: Components of HPLC

The choice of the column should be made after a careful consideration of the mode of the chromatographic technique. Three types of column are available based upon types of packing and particle size, namely, rigid solid, hard gels, and porous and peculiar layer beds, the column of smaller particles (3-10µ) are always preferred because they offer high efficiency(number of theoretical plates /meter) and speed of analysis.

The different types of detection used in HPLC method based on ultraviolet (UV), fluorescence, refractive index, mass spectrophotometric detector or a diode array detector (DAD) Chemical derivatisation procedure for HPLC are performed in order to improve detectability, to improved selectivity (or specificity), to modify the chromatographic properties, and in some cases to provide favorable mass spectral fragmentation pattern for structural elucidation when a mass spectrometer is used either as an off or on line detector.

Approaches to HPLC derivatisation involve both pre and post column methodology. In pre column procedures, derivatisation occurs in vitro before injection of the sample. In post column procedure, the reaction occurs after column separation by adding reagent to the mobile phase and /or by utilizing thermal, UV, catalytic or other effects. Among the chemical reagent employed in precolumn procedure are dansyl chloride, NBD chloride, o-phthaldehyde, and fluorescamine for drugs containing amino group, danshydrazine, NBD-hydrazine, and phenyl hydrazine for keto groups and aldehydes and p-nitro benzoyl chlorides for hydroxyl groups. Almost all of these reagents provide highly fluorescent derivative (called fluorophors) of a drug or metabolites except for phenyl hydrazine and p-nitrobenzoyl chloride, which are used to create a strong absorbing chromophores for better detection of a compound in the UV or visible region.

When a drug or metabolite is difficult to derivatise but possess reasonable Lewis acid or base properties, an ion pair reagent is added to the mobile phase to form an ion–pair with compound, thereby enhancing detection and chromatographic properties. Ion pair technique such as this can be approached with both pre and post–column methodology. Both chromogenic and fluorescing counter ions can be employed depending on the sensitivity requirements of the assay.

Methods for analyzing drug in biological sample can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character, and the solubility parameter. An exact recipe for HPLC

however cannot be provided because method development involves considerable trial and error procedure. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reverse phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration (acetonitrile or methanol) can be increased up to 100% within 20-30 min. Separation can then be optimized by changing the initial mobile phase composition and slope of gradient according to the chromatogram obtained from preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition.

Elution of drug molecules can be altered by changing the polarity of mobile phase. The elution strength of mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic sample (acidic or basic) can be separated; if they are present in un-dissociated form. Dissociation of ionic sample may be suppressed by proper selection of pH.

The pH of mobile phase has to be selected in such a way that the compounds are not ionized. If the retention time is too short, the decrease of mobile phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increased in 5% steps of the organic phase concentration is needed.

Whenever acidic or basic are to be separated it is strongly advisable to control mobile phase pH by adding a buffer, pH of the buffer be adjusted before adding organic phase. The buffer selected for a particular separation should be used to control pH over the range of ≈ pKa ± 1.0 the buffer should transit light at or below 220 nm so as to allow low UV detection.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that the entire compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change in mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, the position of the peaks can be predicted within the

range of investigated changes. An optimizing chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with high theoretical plate number, N), which can be achieve by using a column of smaller particle size, or a longer column these factor, however will increased the analysis time. Flow rate does not influence the resolution, but it has a strong effect on the analysis time.

The parameter that are affected by the changes in chromatographic condition are,

- Resolution (R_s)
- Capacity factor($K^{'}$),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry Factor (As).

High-performance liquid chromatography [HPLC] is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify and quantitated the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be and has been applied to just above any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples and industrial chemicals.

Mobile phases used in HPLC

In HPLC a broad variety of mobile phases can be used. According to the mode of HPLC mobile phase can be selected like polar nature or non-polar. Mobile phase selection depends on many factors like purity, sample solubility, chemical inertness, detector compatibility and low viscosity. Mostly organic solvents like acetonitrile, methanol, ethanol, formic acid etc are used. Sometimes these solvents are mixed with water and also can be used. These mobile phases should be selected according to the properties of drug to be estimated in HPLC, so its selection should be done carefully. Based on the mobile phase only the drug elutes and chromatogram of that drug comes properly.

LISTS OF STEPS NEEDED BEFORE ANY RUN BY HPLC [14]:

- ✓ Filter the solvents with membranes with cut off 0.22-0.45m.
- ✓ Precipitates and colloids can be distinguished by using clean and transparent reservoirs.
- ✓ Make sure that the solvents will be easily mixed with the previous solvents in the same inlets. For example methanol or water should not be placed instead of hexane directly, or any organic solvent should not be placed directly instead of a buffer reservoir.
- ✓ Degas the solvents and remove by cleansing all the tubing that leads to the pump.
- ✓ Connect the column according to the flow direction indicated on it (do not connect directly to the detector).
- ✓ At a low flow rate stream the appropriate the solvents through the column (0.1-0.5 ml/min) or reach the composition gradually using the appropriate gradient.
- ✓ Wait for a stable baseline and select an appropriate wavelength in the detector.
- ✓ Prepare the set of methods in the workstation: Instrument method for the control on the system, processing method for the data processing and the report method for the report of final results.
- ✓ A blank run should be performed to test the system and verify that it is clean from interferences when the methods are ready.

Minimum requirement for HPLC: [15]

A).Temperature:

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure are enhancing selectivity. Typically, temperatures in excess of 60^{o}C are not used.

B).Retention time mechanism:

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bead, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

- Hydrophobic (non-specific) interactions are the main ones in Reversed-Phase Separations.

- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion-exchange Chromatography. All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface and the weaker the eluent interaction, the longer analyte will be retained on the surface.

Significance of drug analysis in body fluids:

Bioanalytical studies have many important applications in pharmacy field. They are mainly:

- Bioequivalence studies.
- Forensic and toxicological studies.
- Drug –drug interaction and Bioavailability.
- Therapeutic drug monitoring (TDM).
- Adverse effect.
- Over dose.
- Drug abuse in sports.
- Pharmacokinetic studies and In clinical trials.

Quantitative Analysis by HPLC [16]:-

Three methods are generally used for quantitative analysis in HPLC. They are the external standard method, the internal standard method and standard addition method

❖ ***External standard method***

The external standard method involves the use of single standard or up to three standard solutions. The peak area or the height of the sample and the standard used are compared directly or the slope of calibration curved based on standards that contain known concentration of the compound of interest.

❖ ***Internal standard method***

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fix concentration is added to the known amount of sample to give separate peak in the chromatograms, to compensate for the losses of the compound of interest during sample pretreatment steps. Any loss of the compound of interest will

be accompanied by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously depends on the structural equivalence of the compounds of interest and the internal standard.

The requirement for an internal standard must

- ✓ Give a completely resolved peak with no interferences
- ✓ Elute close to the compound of interest
- ✓ Behave equivalent to the compound of interest for analysis like pretreatment, derivative formation, etc.
- ✓ Be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound of interest.
- ✓ Not be present in original sample
- ✓ Be stable, unreactive with the sample components, column packing and the mobile phase and
- ✓ Be commercially available in high purity

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it .Response factor is used to determine the concentration of a sample component in the original sample. The response factor (Rf) is the ratio of peak area of sample component (A_x) and the internal standard (A_{IS}) obtained by injecting the sample quantity.

❖ ***Standard addition method:***

In this method for the sample solution known amount of standard is added. By plotting calibration curve graphically the quantitative analysis is carried out. This method is used for sample which contains which contains very small quantity of element to be analyzed. In trace analysis also this method is used. By adding the standard to sample solution the peak area is increased and concentration of sample will be computed by interpretation.

Validation:

Validation is a process of stabling documented evidence, where high degree of assurance can be provided, that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics[17].

Validation is very important for a method. This has to be compulsory done after the method development. In order, to prove that process is consistently doing

1. What it is supposed to do.
2. To determine the process variable and acceptable limits for those variables and to set up appropriate in- process control.
3. To assure the quality and to see that product is prepared in the reasonable amount of time.

Although there are various stages in the development and validation of an analytical procedure, the validation of the analytical method can be envisaged to be to consist of two distinct phases:

a) The ***pre-study phase*** which comes before the actual start of the study and involves the validation of the method on biological matrix human plasma samples and spiked plasma samples.
b) The ***study phase*** in which the validated bioanalytical method is applied to the actual analysis of the samples from bioavailability and bioequivalence studies mainly to confirm the stability, accuracy and precision.

Bioanalytical method validation (BMV) *(18)*

For quantitative determination of drugs and their metabolites in the in biological matrix bioanalytical method validation (BMV) is done and this gives information about various things like pharmacokinetic, bioequivalence, bioavailability, toxicology studies. For regulatory filings these studies will support. It is necessary to emphasize well-characterized and fully validated analytical methods to produce reliable results that can be satisfactorily interpreted. For each analytical method has its own characteristics which may vary from analyte to analyte in these instances. So, specific validation criteria is essential, to be developed for each analyte. Most of the procedures, principles, and requirements for quantitative bioanalytical method validation are common to all types of analytical methods. In bioanalytical method validation two phases are present. They are:

1. The bioanalytical method development phase in which the assay is defined and validated.
2. The application to actual analysis of samples from pharmacokinetic, bioavailability, bioequivalence, and drug interaction studies.

In bioanalytical method validation (BMV) validation is done by three different levels/types of validation are done. They are Full validation, Partial validation, Cross validation.

Full validation:

It is done when developing bioanalytical method for first time for a new drug. Full validation is performed when an existing assay for quantification was added by metabolites.

Partial validation:

These are modifications to bioanalytical methods for which Full validation is not necessary. Modifications done for bioanalytical methods such as, small changes like change in species with matrix (from rat plasma to mouse plasma),change in matrix with in a species (from human urine to human plasma) change in laboratories or analysts, instruments, change in sampling process procedures, change in analytical method like changing detector.

Cross validation:

In this two bioanalytical methods are compared. The "reference" method which is original one is compared with the revised one "comparator". This is done where two bioanalytical methods are compared and from that same data is prepared for study. This is done in two ways. Spiked matrix samples and subjected samples validation done at sane site or done at different sites i.e. different laboratories or by using different techniques in same laboratory.

FDA guidance on bioanalytical method validation [19]:

For bioanalytical method validation FDA had given some guidelines. These guidelines are given the validation performed was to be accurate. They are:

- Analyte stability in biological matrix at intended storage and operating conditions should be kept.
- Standard curve for matrix-based one should contain minimum 5 standards without including blank and it should cover the entire range of concentrations expected.

- Essential parameters that are performed for acceptability of bioanalytical method are precision, accuracy, selectivity, sensitivity and reproducibility.
- For lowest concentration on the standard curve the lower limit of quantification can be used and that is not confused with limit of detection.
- During the Full validation of a bioanalytical method the below parameters should be defined.

Validation parameters [20]:

Different Types of Validation characteristics:

- Precision
  - Repeatability
  - Intermediate Precision
  - Reproducibility
- Accuracy
- Specificity Selectivity
- Linearity Range
- Limit of Detection (LOD)
- Lower Limit of Quantification (LLOQ)
- Robustness
- Ruggedness.
- System Suitability

1) Precision:

When the procedure is applied repeatedly to multiple samplings of single homogenous sample under prescribed conditions then precision, is a closeness of individual measurements of the analyte. It is done at three levels such as repeatability, intermediate precision, and reproducibility.

Repeatability: It expresses precision under same operating conditions i.e. with in the laboratory same analyst using same equipment over a short period of time.

Intermediate precision: It is the precision under different laboratory conditions i.e. varying only in different analyst, on different days, or using different equipments within the same laboratory.

Reproducibility: It is the precision between different laboratories and is often determined in method transfer experiments

Acceptance Criteria:

- ✓ Percentage Relative deviation (%RSD) NMT 1 % (Instrument precision)
- ✓ (%RSD) NMT -2% (Intra- assay precision)

2) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Most commonly used method for determination of accuracy is recovery studies. The usual range is being 10% above or below the expected range of claim. The % recovery was calculated using the formula,

$$\%\,\mathrm{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

Acceptance Criteria:

- In assay method, mean recovery will be 100% ± 2% at each concentration between the ranges of 80-120% of the target concentration.
- In impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities between the ranges of 0.1-2.5 % (V/W).

3) Limit of detection (LOD):

The limit of detection is the lowest concentration of analyte in the sample which can be detected but not quantified under given experimental conditions. The lowest concentration which can be distinguished from the background noise with a certain degree of confidence is defined as limit of detection. Prepare the blank solution as per test method and inject six times into the chromatographic system. Similarly prepare the linearity solution staring from lowest possible concentration of

analyte to 150 % (or as per protocol) of target concentration and establish the linearity curve.

The detection limit (DL) may be expressed as:

$$LOD = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

4) Lower Limit of quantification (LLOQ):

It is also the lowest concentration of analyte in the sample but quantitatively determined with suitable accuracy and precision.

In calibration curve it is the lowest concentration point. It is determined by accuracy by the presence of background signal and by precision i.e. reproducibility of analyte in the method.

$$LOD = \frac{10 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.
- LOQ defined as the lowest concentration providing a RSD of 5%.
- LOQ should be at least 10% of the minimum effective concentration for clinical applications.

5) Specificity:

A method is said to be specific when it produces proper response only for a single analyte. It can be demonstrated by performing Placebo / blank interference and forced degradation studies. If the expected impurities or related substances are available, then they should be analyzed along with the analyte or sample to check the system suitability, retention factor, tailing factor and resolution etc. In this peak purity studies are done for specificity.

6) Linearity:

As per ICH definition "the ability to obtain test results which are directly proportional to the concentration of an analyte within given range is known as linearity of an analytical procedure". By using correlation coefficient this can be tested. Using correlation coefficient is a benefit as it is a relationship between concentration and response data. In this data is analyzed by linear least square regression co-efficient and b of the linear equation,

$$Y = aX + b$$

By the above equation regression **r** value can be known. For the method to be linear the **r** value should be close to1. Where **Y** is the measured output signal, **X** is the concentration of sample, **a** is the slope, **b** is the intercept.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

7) Robustness:

It is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

8) Ruggedness:

Ruggedness according to the USP is "the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, and different lots of reagents. The following are the typical method parameters need to test during method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability.
- On different days.
- In different laboratories.

APPLICATION OF A VALIDATED BIOANALYTICAL METHOD TO ROUTINE DRUG ANALYSIS [21]

Many of the above principles under method establishment and validation are relevant to within study validation. This section will emphasize the validation parameters that should be evaluated during routine application of a validated bioanalytical method to a particular study. Following a successful validation which meets an *a prior* set acceptance criteria for accuracy and precision, examination of biological samples can be done by single determination without a need for duplicate or replicate analysis. The need for duplicate analysis may arise for special cases. For example, in the case of a difficult procedure for labile analyte (s), when the precision and accuracy tolerances are difficult to achieve, duplicates analyses may be essential.

A matrix-based standard curve should be generated for each analytical batch for each analyte and should be used in calculating the concentration of analyte in the unknown samples assayed with that run. It is important to use a matrix based standard curve that will cover the entire range of concentrations in the unknown samples. Estimation of unknowns by extrapolations of standard curve below the LLOQ or above the ULOQ is not recommended. Instead, it is recommended that the standard curve be re-determined or samples be reassayed after dilution with the matrix.

- ✓ A matrix-based standard curve should consist of a minimum of five to eight standard points, excluding blanks (either single or replicate), covering the entire range.
- ✓ Response Function: Typically, the same curve fitting, weighting and goodness of fit determined during pre-study validation should be utilized for the standard curve within study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation.
- ✓ The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- ✓ System suitability: Based on the analyte and technique, a specific standard operating procedure (or sample) can be identified to assure the optimum operation of the system employed.
- ✓ Any required sample dilutions must utilize like matrix (e.g. human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.

Table No 2: Method Validation Requirements for Example (ICH)

METHOD VALIDATION REQUIREMENTS	ACCEPTANCE CRITERIA
Precision	
Assay repeatability	≤ 1% RSD
Intermediate precision (Ruggedness)	≤ 2% RSD
Accuracy	
Mean recovery per concentration	100.0% ± 2.0%
Limit of detection	
Signal to-to-noise ratio	≥ 3:1
Limit of quantification	
Signal to-to-noise ratio	≥ 10:1
Linearity/Range	
Correlation coefficient	>0.99
y-Intercept	± 10%
Visual	Linear
Robustness	
System suitability met	yes
Solution stability	± 2% change from time zero
Specificity	
Resolution from main peak	>2 min. (retention time)

2. LITERATURE REVIEW

1) **R.Rajameena** *etal*[22] Developed and Validated for the estimation of Clindamycin phosphate and Clotrimazole in pharmaceutical dosage forms using Reverse Phase High Performance Liquid Chromatographic Method. The elution was done using a Hypersil BDS C8 (250 × 4.6 mm; 5µm) column using mobile phase consisting of Phosphate buffer pH 2.5 and Acetonitrile in the ratio of 70:30 was used. The detection wavelength was 210 nm and the flow rate was 1.0 ml/min. The linearity was found in the range of 80-120 mg/ml. The method was successfully applied to the pharmaceutical formulation because no chromatographic interferences from peccaries excipients were found.

2) **Amit V Patel** *etal*[23] Developed and Validated reversed‐phase HPLC method for simultaneous estimation of Clotrimazole and Beclomethasone dipropionate in lotion and cream dosage form. In this method, the chromatographic separation was achieved on a Kromasil C18 (150 mm × 4.6 mm, 5 µm) analytical column. A mixture of acetonitrile–water (70:30, v/v) was used as the mobile phase, at a flow rate of 1 ml/min and detector wavelength at 254 nm. The retention time of CT and BD was found to be 5.4 and 4 min, respectively. The linear dynamic ranges were from 2-16 µg/ml and 80-640 µg/ml for BD and CT, respectively. The developed method was validated and found to be simple, specific, accurate and precise and can be used for routine quality control analysis of titled drugs in combination in lotion and cream formulation.

3) **Silveira** *etal*[24] Developed and Validated a simple Reverse phase- HPLC Method for the assay of Clotrimazole loaded nanocapsule suspensions. The chromatographic conditions were RP Phenomenex column (250 x 4.60 mm, 5 µm particle size) using mobile phase methanol-water (90:10) at isocratic flow rate of 1mL/min, injection volume at 20 µL and the detection was performed at 229 nm. The proposed method is specific, precise, accurate, linear for the concentration range of 2.0-10.0 mg/mL and robust.

4) **Renata Hajkova** *etal*[25] Developed and Validated HPLC method for determination of Clotrimazole and its two degradation products in spray formulation. In this method, the

determination of three compounds in spray solution (active component Clotrimazole and two degradation products imidazole and (2-chlorophenyl)diphenylmethanol) using ibuprofen as an internal standard was developed and validated. Reversed-phase chromatography was performed on Zorbax® SB-Phenyl column (75 mm × 4.6 mm, 3.5 µm) using mobile phase for separation of clotrimazole, degradation products imidazole and (2-chlorophenyl)diphenylmethanol and ibuprofen as internal standard consists of a mixture of acetonitrile and water (65:35, v/v) with pH conditioned by phosphoric acid to 3.5. At a flow rate of 0.5 ml min^{-1} and detection at 210 nm, the total time of analysis was less than 6 min.

5) **Dr. Adnan Manassra** *etal*[26] reported HPLC method for Simultaneous estimation of Betamethasone and Clotrimazole in Cream Formulations. The elution was done using a mobile phase consisting of methanol-acetate buffer pH 6.8 -acetonitrile (33:27:40, v/v) on C18 (250 X 4.0 mm) stationary phase and spectrophotometric UV detection at 254 nm. The linearity range was found 0.025 to 0.075 mg/ml for betamethasone and Clotrimazole.

6) **FrankWienen** *etal*[27] reported a capillary electrophoretic method for estimation of Clotrimazole in mice plasma. Sample preparation was carried out by protein precipitation using methanol. Quantification of Clotrimazole was achieved by means of capillary electrophoresis using Ketoconazole as an internal standard (IS). The background electrolyte (BGE) composed of a Tris buffer solution (100 mM, pH 3.0, adjusted with acetic acid) and methanol (8:2, v/v). Injection was carried out electro kinetically with 10 kV over a time period of 20s. A special rinsing procedure utilizing a sequence of a SDS/methanol solution, a sodium hydroxide solution, water and BGE, was applied to enhance the reproducibility. With this procedure, an intermediate precision (day-to-day precision) of the area ratios of Clotrimazole and IS of 5.0% for 0.5 mg/ml^{-1} and 2.6% for 10 mg/ml^{-1} was obtained.

3. DRUG PROFILE[28]

CLOTRIMAZOLE

3.1 Chemical profile [28]

Brand name:

Candid, Abzorb, Canazole, Clocit VG, Mycelex, Moncid, Oraloz

Chemical name/IUPAC NAME:

1-[(2-chlorophenyl) (diphenyl)methyl]-1*H*-imidazole

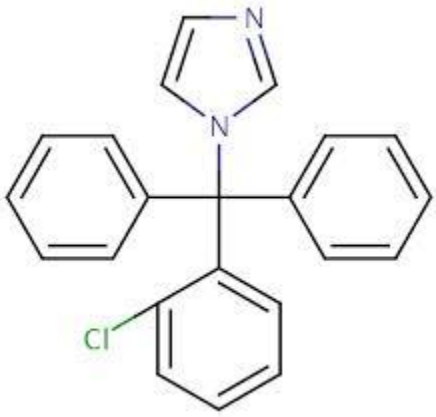
Molecular formula:

$C_{22}H_{17}ClN_2$

Mol. mass: 344.837 g/mol

Structure:

Fig 1: MOLECULAR STRUCTURE OF CLOTRIMAZOLE



Pharmacokinetic data:

Bioavailability	: 10.9 ± 6.8% (oral)
Metabolism	: Hepatic (metabolized to inactive metabolites)
Protein Binding	: 90%
Half-life	: 2 hrs
Excretion	: Not available
Routes	: Topical, Oral

Description:

Clotrimazole is an odorless, white crystalline substance. It is practically insoluble in water, sparingly soluble in ether and very soluble in polyene glycol 400, ethanol and chloroform.

Category [28]:

Antifungal and Inhibitor of Cytochrome P450 Oxidase

Dose [28]:

Each mL of Clotrimazole topical solution contains 10 mg Clotrimazole, USP in a nonaqueous vehicle of polyethylene glycol 400.

Indication:

For the local treatment of Mouth ulcer, oropharyngeal candidiasis and vaginal yeast infections, also used in fungal infections of the skin such as ringworm, athlete's foot, and jock itch.

5.2 Pharmacological profile

Mechanism of action:

Clotrimazole interacts with yeast 14-α demethylase, a cytochrome P-450 enzyme that converts lanosterol to ergosterol, an essential component of the membrane. In this way, clotrimazole inhibits ergosterol synthesis, resulting in increased cellular permeability. Clotrimazole may also inhibit endogenous respiration, interact with membrane phospholipids, inhibit the transformation of yeasts to mycelial forms and the uptake of purine, impair triglyceride and/or phospholipid biosynthesis, and inhibit the movement of calcium and potassium ions across the cell membrane by blocking the ion transport pathway known as the Gardos channel.

Pharmacodynamics

Clotrimazole action leads to increased membrane permeability and apparent disruption of enzyme systems bound to the membrane. In pharmaceutical formulation, Betamethasone and Clotrimazole are used together to treat cutaneous tinea infections. In

studies of fungal cultures, the minimum fungicidal concentration of clotrimazole caused leakage of intracellular phosphorous compounds into the ambient medium with concomitant breakdown of cellular nucleic acids, and accelerated potassium etflux. Both of these events began rapidly and extensively after addition of the drug to the cultures. The primary action of clotrimazole is against dividing and growing organisms.

Drug Interactions:

The limited therapeutic arsenal and the increase in reports of fungal resistance to multiple antifungal agents have made fungal infections a major therapeutic challenge. Clotrimazole Antagonises with polyene antibiotics. The polyene antibiotics like Natamycin inhibits growth of yeasts and fungi via the immediate inhibition of amino acid and glucose transport across the plasma membrane. This is attributable to ergosterol-specific and reversible inhibition of membrane transport proteins. Therefore polyene antibiotics interferes with Clotrimazole administration.

Adverse reactions:

The following adverse reactions have been reported in connection with the use of Clotrimazole and symptoms of overdose include erythema, stinging, blistering, peeling, edema, pruritus, urticaria, burning, and general irritation of the skin, and cramps.

Contraindications:

It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Clotrimazole is used by a nursing woman.

Safety and effectiveness in pediatric patients have been established for Clotrimazole when used as indicated and in the recommended dosage.

4. AIM AND OBJECTIVE

Estimation of drugs in biological media is increasingly important nowadays, which reveals information like bioavailability, bioequivalence, drug abuse, and pharmacokinetics and drug research.

HPLC is the most suitable technique for the analysis of biological fluids owing to its well-developed characteristics and ruggedness. It is an extremely sensitive, precise, accurate, and rapid, separation technique.

Clotrimazole is a broad spectrum antifungal agent that is used for the treatment of infections caused by various species of pathogenic dermatophytes, yeasts, and *Malassezia furfur*. The primary action of Clotrimazole is against dividing and growing organisms. Clotrimazole interacts with yeast 14-α demethylase, a cytochrome P-450 enzyme that converts lanosterol to ergosterol, an essential component of the membrane. In this way, Clotrimazole inhibits ergosterol synthesis, resulting in increased cellular permeability.

Few analytical methods were reported for the quantitative determination of Clotrimazole and its combination with other drugs by HPLC. Only one method was reported for Clotrimazole in mice plasma by capillary electrophoresis. But there is no RP-HPLC method reported for the bio-analytical method development in human plasma using protein precipitation method under different chromatographic conditions.

The present study is planned to develop newer analytical method for the determination of Clotrimazole by bio-analytical method development in human plasma using protein precipitation by RP-HPLC under different chromatographic conditions.

The reasons for determination of Clotrimazole Bio-Analytical method by RP-HPLC are as follows:

- To develop new RP-HPLC Method by Isocratic mode.
- To reduce the run time.
- To carry out estimation of Clotrimazole by using different chromatographic conditions.
- To develop a new procedure for protein precipitation method.

Advantages of less run time in HPLC:

- It's beneficial to the company economically.
- To estimate the different compounds with less time in different formulations like tablets, capsules, syrups, expectorants and injections.
- Utilisation of minimum solvent.
- Reduce the cost.
- Less utilisation of men, machine and materials.

5. PLAN OF WORK

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD:

Bioanalytical method development for Clotrimazole:

The present work is planned to divide into three phases:

- **PHASE I**

1. Optimization of chromatographic conditions

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Selection of internal standard
- Sample preparation by protein precipitation method
- Estimation of clotrimazole

- **PHASE II**

2. Validation of the method

The developed method were proposed to be validated using the various validation parameters such as,

- Accuracy
- Precision
- Limit of detection (LOD)
- Lower Limit of quantitation (LLOQ)
- Selectivity / Specificity
- System suitability
- Ruggedness

6. METHODOLOGY

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Materials and Instruments used

Drug sample and Study products

Clotrimazole was obtained from Hetero Healthcare Ltd, Hyderabad, India.

Ibuprofen was obtained from Hetero Healthcare Ltd, Hyderabad, India.

Plasma

Blank plasma was collected from Kovai Medical Center and Hospitals, Coimbatore.

Chemicals and solvents used for estimation-

HPLC Water	-	Qualigens, Mumbai, India.
Acetonitrile	-	Himedia, Mumbai, India.
Methanol	-	Himedia, Mumbai, India.
Distilled water	-	Double Distilled water.
Potassium Di-hydrogen phosphate	-	Analytical grade, SD fine chemicals, Mumbai.
Orthophosphoric acid	-	SD fine chem. Ltd, Mumbai.
Triethylamine	-	Analytical grade, SD fine chemicals

Instruments used:

- ✓ Elico pH meter LI 127.
- ✓ Shimadzu LC-20 AT HPLC.
- ✓ SPD-M20A Prominence diode array detector.
- ✓ Shimadzu 1700 LC-UV Spectrophotometer.
- ✓ Sonica ultrasonic cleaner.
- ✓ Solvent filtration unit – Millipore.
- ✓ Shimadzu electronic balance AY 220.
- ✓ Ultra cooling centrifuge – Remi, India

OPTIMIZATION OF CHROMATOGRAPHIC CONDITION FOR THE ESTIMATION OF CLOTRIMAZOLE [37]

Selection of Wavelength-

An uv spectrum of 100 µg/ml Clotrimazole in methanol was recorded by scanning in the range of 200 nm to 400 nm. A wavelength which gives good response for the drugs to be detected is to be selected. From the UV spectrum a wavelength of 215 nm was selected. Clotrimazole showed maximum absorbance at this wavelength.

Selection of Chromatographic Method

Selection of proper chromatographic method depends on the nature of the sample or its properties like ionic/ionizable/neutral character, its molecular weight and solubility. The drug selected for the present study was polar in nature hence, reverse phase HPLC or ion-pair or ion–exchange chromatography method must be used. Because of its simplicity and suitability for initial separations reverse phase method was selected.

Initial chromatographic conditions for separation of Clotrimazole

Standard solution:

100µg/ml of Clotrimazole was prepared by dissolving in 10 ml of HPLC grade Clotrimazole.

Equipment

System : Shimadzu gradient HPLC

Pump : LC-20AT prominence solvent delivery system

Detector : SPD-M20A prominence diode array detector

Injector : Rheodyne 7725i with 20µl loop

Chromatographic conditions – 1

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-Water
		Solvent B-Methanol
Solvent Ratio	:	50:50
Detection wavelength	:	215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

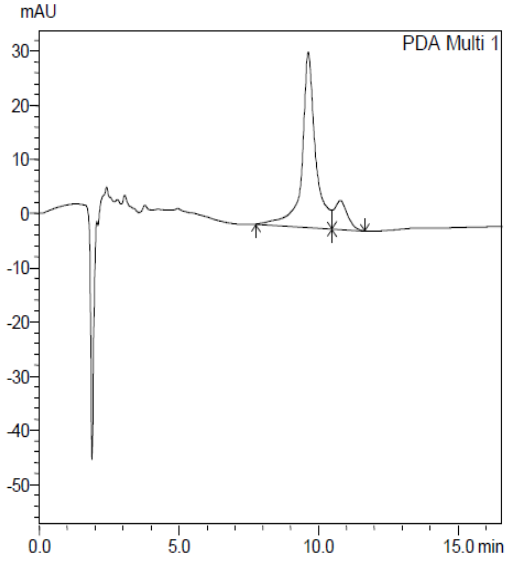


Fig 8 - Chromatographic conditions – 1

At the above chromatographic conditions Clotrimazole was eluted at the retention time of 9.8 min. The peak observed was fronting and splitting, thus not selected further for studies.

Chromatographic conditions – 2

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-Water
		Solvent B-Acetonitrile
Solvent Ratio	:	60:40
Detection wave length	:	215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

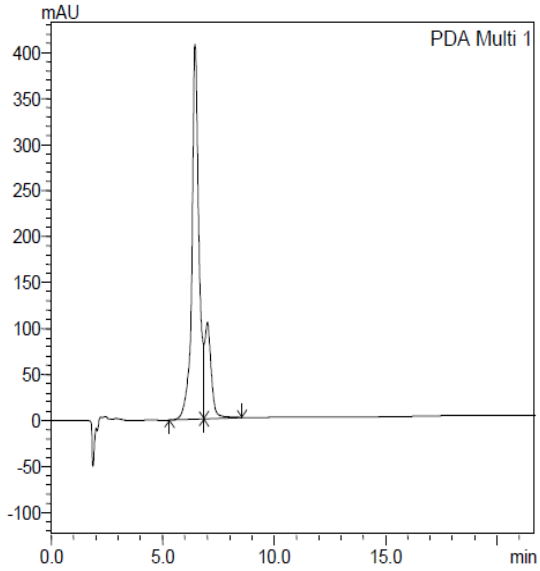


Fig 9 - Chromatographic conditions – 2

Clotrimazole was eluted at retention time of 6.2 minute with peak splitting and tailing, hence not selected further for method development.

Chromatographic conditions – 3

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-0.5% TEA in water pH adjusted to 5.5 (with Orthophosphoric Acid) Solvent B-Acetonitrile
Solvent Ratio	:	30:70
Detection wave length	:	215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

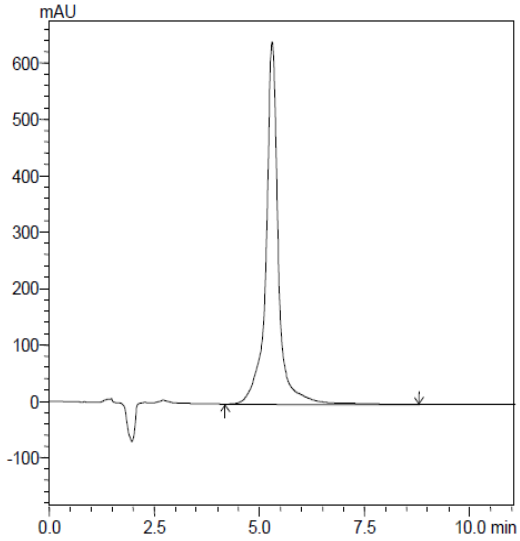


Fig 10 - Chromatographic conditions – 3

Clotrimazole was eluted at retention time 5.5 minutes with peak fronting and peak tailing, hence not selected further for method development.

Chromatographic conditions – 4

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-0.5% Triethylamine in Water pH adjusted to 4.5 (with Orthophosphoric Acid) Solvent B-Acetonitrile
Solvent Ratio	:	40:60
Detection wave length	:	215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

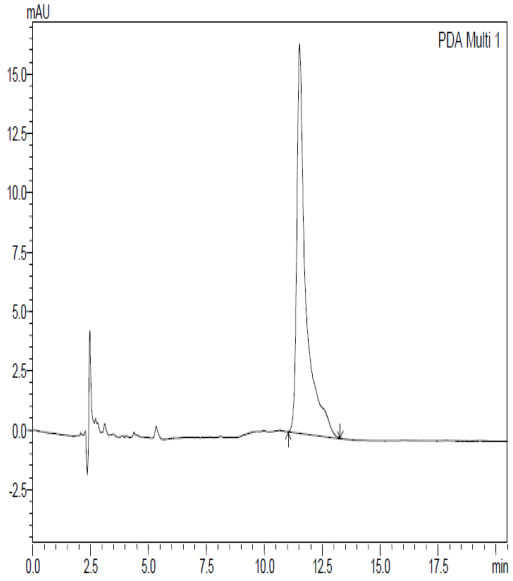


Fig 11 - Chromatographic conditions – 4

Clotrimazole was eluted at retention time of 11.3 minutes with peak tailing, and hence, not selected further for method development.

Chromatographic conditions – 5

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-0.5% Triethylamine inWater pH adjusted to 3.5 (With Orthophosphoric Acid) Solvent B-Acetonitrile
Solvent Ratio	:	35:65
Detection wave length:		215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

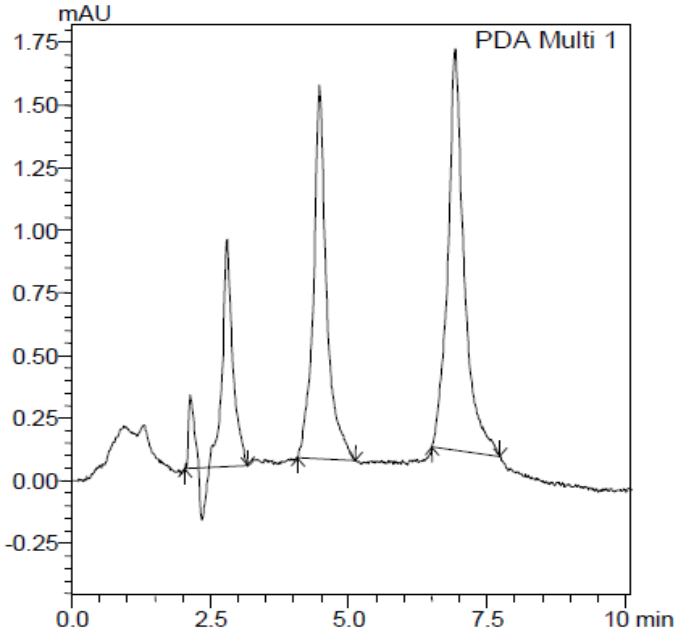


Fig 12 - Chromatographic conditions – 5

Clotrimazole was eluted at retention time of 7.0 minutes and 4.5 for IS with peak fronting, and tailing hence not selected further for method development.

Fixed Chromatographic Conditions – 6

Stationary phase	:	Phenomenex C18 column
Solvent Phase	:	Solvent A-0.5% Triethylamine in Water pH adjusted to 3.0 (with Orthophosphoric Acid) Solvent B-Acetonitrile
Solvent Ratio	:	20:80
Detection wave length:		215 nm
Flow rate	:	1 ml/min
Injection volume	:	20µL
Column Temperature	:	Ambient

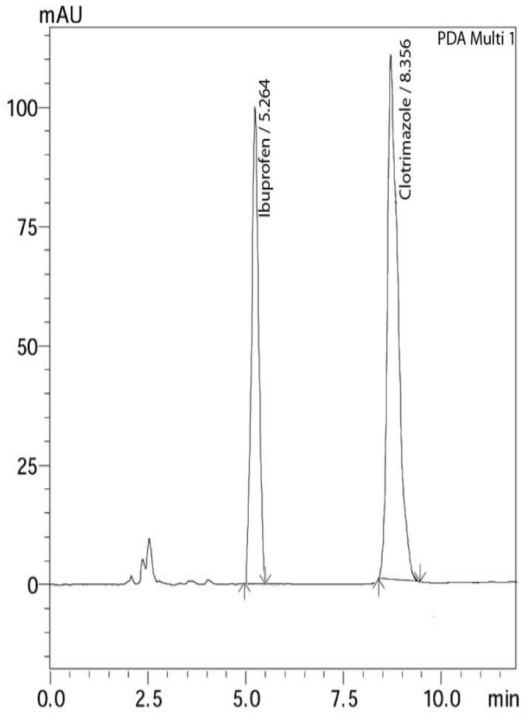


Fig 13 - Chromatographic conditions – 6

Clotrimazole was eluted at 8.3 mins and internal standard ibuprofen at 5.2 with perfect peak properties, hence selected for further studies.

Preparation of standard solution of clotrimazole:

Ten mg of standard Clotrimazole was weighed and dissolved in mobile phase and made upto 10 ml in a volumetric flask. It was further diluted with mobile phase to obtain the concentration in the range of 10-50 μg/ml.

Effect of pH

Table 1. Effect of pH on development of chromatogram

Drug	Ph	Retention time (min)	Observation
Clotrimazole	5.5	5.5	Fronting & Tailing
	4.5	11.3	Tailing
	3.5	7.0	Tailing
	3.0	8.3	Good

Effect of ratio of mobile phase

The mobile phase (0.5% Triethylamine in water adjusted to 3 and acetonitrile) in the ratio of 40:60, 30:70, 20:80 was tried and the chromatogram was recorded at 215 nm with a flow rate of 1ml/min. At 20:80 ratios symmetric peaks were eluted at 5.3 and 8.3 for internal standard and Clotrimazole respectively. At 40:60 and 30:70 peaks were asymmetrical in shape. Thus for the present study 20:80 ratio of (0.5% Triethylamine in water) and acetonitrile was selected as the mobile phase.

Effect of flow rate

Keeping the mobile phase ratio at 20:20, v/v 0.5% Triethylamine in water: acetonitrile, the chromatograms were recorded at a flow rate of 0.5ml/min, 1.0ml/min 1.5ml/min. At flow rate

of`1.0ml/min, the peaks were sharp and separated with good resolution. Hence, 1ml/min was kept constant for the present analysis.

Selection of internal standard

Based upon polarity and solubility, Albendazole, Neproxin, and Ibuprofen were selected and chromatographed along with the standard drug. The elution time of Ibuprofen was 5 min. The peak of ibuprofen was symmetric and well resolved from the peak of the Clotrimazole. Hence, for the present study ibuprofen was selected as the internal standard.

Pretreatment method for biological fluid:

Method of sample preparation is an important criterion for biological samples. For the present study plasma was obtained from clinical laboratory services of KMCH hospital, coimbatore. Protein precipitation method was selected for the present study.

Protein precipitation

This method is the most commonly used method for the extraction of analytes from biological fluids. In this method precipitating agents like acetonitrile, methanol and mixture of acetonitrile-methanol etc was added to the blank plasma to precipitate the proteins present in the plasma and then the precipitate formed was removed by filtration or centrifugation. The supernatant resulted is directly injected into HPLC column without any further treatment and chromatograms were recorded.

An aliquot of 0.1ml of plasma in a glass tube 0.05 ml of standard solution, 0.1 ml of internal standard solution and 0.25 ml of mixture of acetonitrile-methanol in the ratio of (80:20,v/v) was added and then centrifuged for 15 minutes at 5000 rpm. The same extraction procedure was also repeated by using methanol and acetonitrile separately for extraction of drug from plasma and the percentage recovery was calculated for all the precipitating agents.

The chromatograms of plasma extracted with acetonitrile, methanol and mixture of acetonitrile- methanol (80:20, v/v) were recorded using the fixed chromatographic conditions.

The chromatogram of blank plasma without any drug was also recorded. Based up on the percentage recovery acetonitrile-methanol mixture (80:20, v/v) was selected for the present study because of its higher percentage recovery.

Preparation of standard stock solution:

Stock solution of Clotrimazole and internal standard 1mg/ml were prepared separately by dissolving 10mg of each drug in 10ml standard flasks and the volume was made up to 10 ml with the mobile phase.

Working standards:

From the stock solution working standard solutions of 100µg/ml was prepared by diluting 1ml to 10ml with mobile phase. Further solutions were made by from the above solution by diluting 0.1ml, 0.2ml, 0.3ml, 0.4ml, and 0.5ml standard solutions to 10ml in a standard flask with mobile phase to get effect concentrations of 1, 2, 3, 4, and 5µg/ml respectively. In the similar way the working standards were prepared for internal standard also.

Preparation of standard graph:

Preparation of calibration standards

To 0.1 ml of blank plasma, 0.05 ml of working standard solution of Clotrimazole and 0.1 ml of internal standard working solutions were added to get concentration of 50, 100, 150, 200 and 250ng/ml respectively. To these calibration standards 0.25 ml of precipitating agent acetonitrile was added and then centrifuged for 15 minutes at 5000 rpm. After centrifugation the clear supernatant liquid was collected and a quantity of 20µl was injected into the HPLC column and chromatograms were recorded. Standard calibration graph was plotted using ratio of peak area of Clotrimazole to its concentration.

ESTIMATION OF CLOTRIMAZOLE IN HUMAN PLASMA:

Recording the chromatogram

The optimized chromatographic conditions were maintained to record the chromatograms of the calibration standards of Clotrimazole and sample from a clinical study. First, baseline stabilization was done for about 20 minutes. Then standard solutions, calibration standard solutions and sample from clinical study containing Clotrimazole were injected and chromatograms were recorded.

VALIDATION OF THE METHOD

After developing a method its validation is necessary to prove the suitability of the method for the intended purpose. Here the procedure followed for the validation of the developed method is described.

a) **Precision*:***

Intraday and interday precision studies were conducted. In intraday precision plasma sample containing drug at three different concentrations with internal standard were injected and chromatogram was recorded. Similarly interday precision over a two week period time was evaluated.

Acceptance criteria:

RSD of the mean concentration of five readings should be less than 15% for bioanalytical method.

b) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. In this studies the selected concentration of the plasma were injected six times and mean peak area for each concentration was calculated. Concentration of the each injection was calculated and the standard deviation between the readings is calculated. To bioanalytical study the percentage RSD should be less than 15%.

c) Recovery studies:

The relative recovery of drug from plasma was calculated by comparing the readings of concentration obtained from the drug spiked plasma to that of equal concentration from standard sample. Recovery studies were carried out six times for sample concentration at three levels within the calibration curve.

Acceptance Criteria:

For an assay method, mean recovery should be 85-105% ± 2%.

d) Linearity and Range:

Linearity and range were estimated by using calibration curve. By using calibration standards prepared by spiking plasma (Clotrimazole) and internal standard (Ibuprofen) at different concentrations like 50ng/ml to 250ng/ml the calibration graph was plotted taking concentration of spiked plasma on x-axis and peak area on y-axis. The linearity is determined from 50% to 250% of the proposed concentration.

Acceptance Criteria:

Coefficient of correlation of the calibration should be not less than 0.99

e) Lower Limit of Quantification (LLOQ):

The LLOQ is determined by using the calibration curve. Limit of quantitation is the concentration of substance in the sample that will give a signal-to-noise ratio of 10:1. Detection limit corresponds to the concentration that will give a signal-to-noise ratio of 3:1. The signal to noise ratio were performed by comparing measured signal of blank plasma sample with those of known low concentration of drug.

f) Specificity:

Specificity of the method was demonstrated by using diode array detector peak purity test. The diode array spectrum of both standard and sample peak were recorded and compared. The other way for doing specificity based in measurement of absorbance ratio of drug peaks at

two different levels. The retention time (Rt), resolution factor (Rs) and tailing (T) were noted for the peaks of Clotrimazole. Peak purity study is done to prove that a developed method is specific for the drug of interest.

Acceptance criteria:

Purity angle should be less than purity threshold i.e.0.99-1.00

h) Selectivity:

Selectivity is the analytical method ability to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity was established by two different methods.

Method I: Chromatograms of six blank plasma samples were compared with chromatogram obtained from standard solutions. Each chromatogram was tested for interferences due to endogenous plasma component on the retention times of the selected drugs.

Method II: This method involves the peak purity test method using diode array detector. The PDA spectrum, UV spectrum, absorbance ratio curve and first derivative spectrum of the standard and sample peaks was recorded using PDA detector and compared for the peak purity of drug.

i) Robustness:

The robustness of the method was studied by changing the chromatographic conditions slightly. The standard solutions were injected in these changed chromatographic conditions.

- ± 1 % difference in the ratio of acetonitrile in the mobile phase.
- ± 0.5 difference in units of pH of the buffer.
- ± 1 % difference in flow rate of the mobile phase.

In these changed conditions the separation factor, retention time and peak symmetry was calculated. Deviation in results from original run should be less than 2%.

j) System suitability studies:

In system suitability studies certain parameters were calculated namely, column efficiency, resolution, capacity factor by repeated injection of standard solutions. As specified in the USP these systems suitability studies were carried out.

Capacity factor (k') it is measurement of sample molecule how good is retained by a column during separation. The ideal k value ranges from 2-10.

$$\text{Capacity Factor (k')} = V_1 - V_0 / V_0$$

Where, V_1 is the retention volume at the apex of the peak (solute) and V_0 is the void volume of the system.

Resolution (Rs) is the difference between the retention times of two solutes divided by their average peak width. The ideal value of (Rs) is 1.5

$$\text{Resolution (Rs)} = Rt_1 - Rt_2 / 0.5(W_1 - W_2)$$

Where, Rt_1 and Rt_2 are the retention times of component 1 and 2, respectively.

Column Efficiency (N) of a column is measured by the number of theoretical plates per meter. For ideal good separation, column efficiency N value ranging from 5,000 to 100,000 plates/meters.

$$\text{Column efficiency (N)} = R_t^2 / W^2$$

Where R_t is the retention time and W is the peak width.

Peak asymmetry factor- For better column performance it was calculated by the formula. When asymmetry factor of value 0.9 to 1.1 then it is achievable for a well packed column.

$$\text{Peak asymmetry factor (As)} = b / a$$

Where a and b are the distances on either side of the peak midpoint.

Table No 2: System Suitability Parameters and Recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD < / = 1 % for N > / = 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T of < / = 2
Theoretical Plates (N)	In general should be > 2000

7. RESULTS AND DISCUSSION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

Bioanalytical method was developed for Clotrimazole and it was validated for its transferability.

Chromatographic separation of standard Clotrimazole:

The chromatogram of Clotrimazole was recorded alone and shown in figure **(Fig4).**The standard solution which contains internal standard Ibuprofen was injected with the developed chromatographic conditions, and the chromatograms were recorded and shown in figure **(Fig 5).**

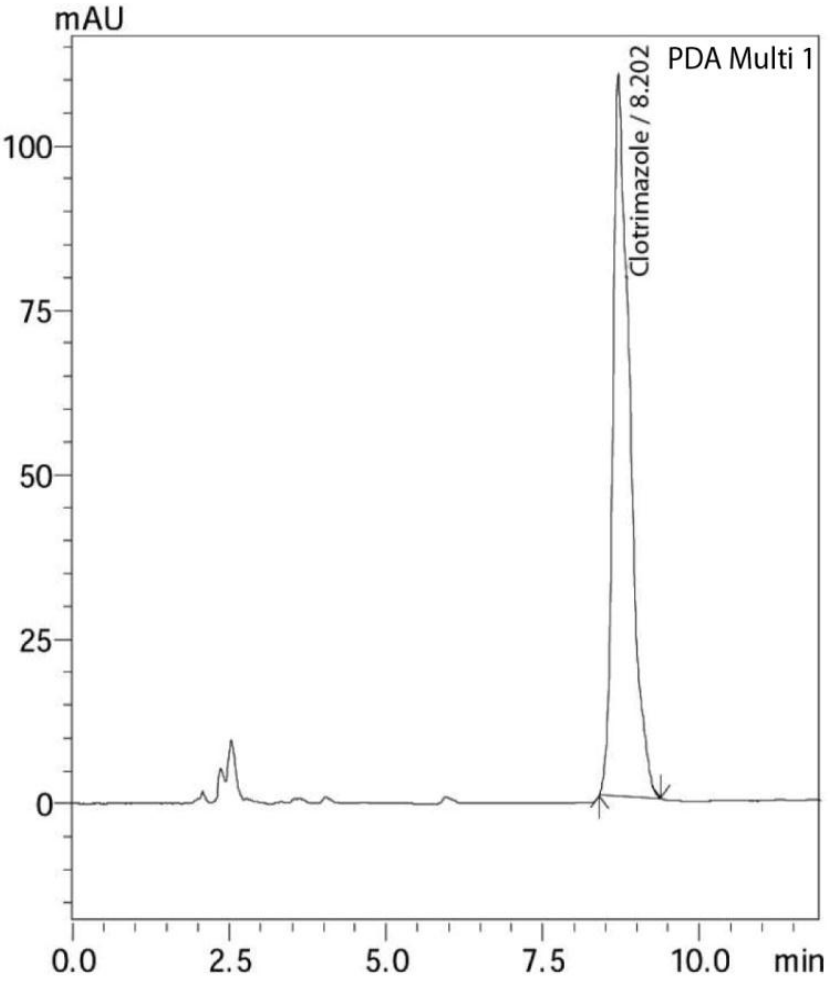


Fig 4 - Chromatogram of standard Clotrimazole 10 µg/ml

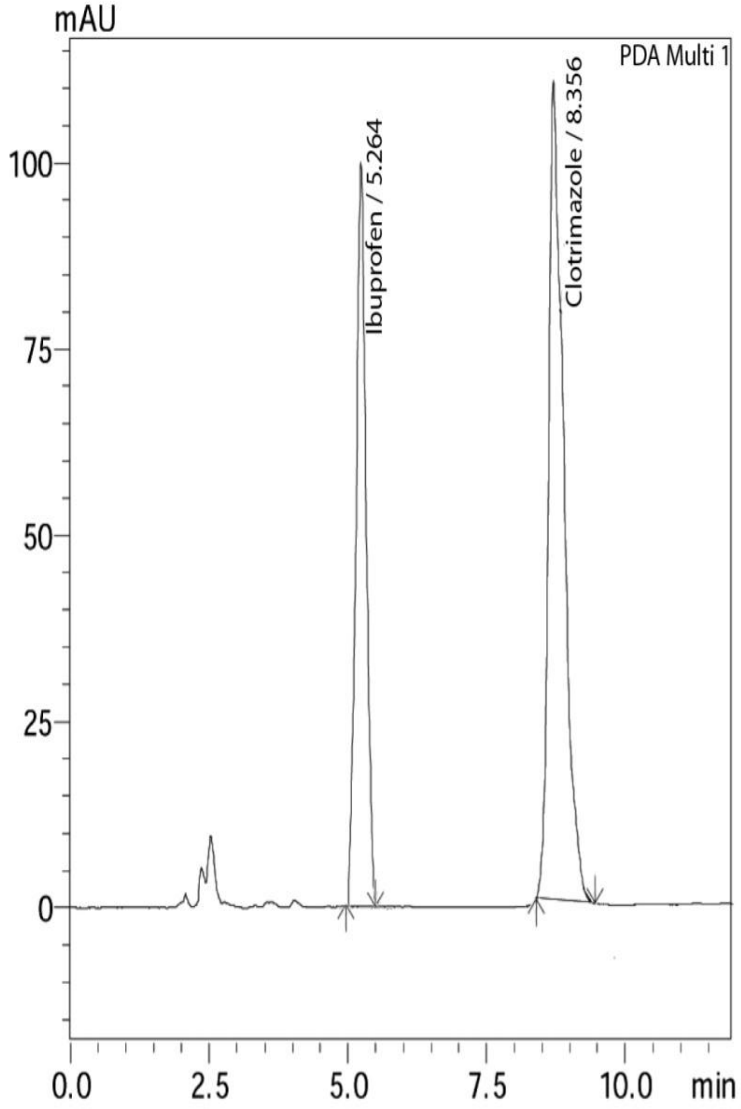


Fig 5 - Chromatogram of Ibuprofen (50 µg/ml) and standard Clotrimazole 10 µg/ml

The retention time of Clotrimazole and internal standard (Ibuprofen) was 8.3 and 5.2 min respectively with percent RSD of less than 2%. The results are shown in table **(Table 1)**. The peak purity study reveals that signal ratios (relative absorbance at different wavelengths) were constant across the peak profile of Clotrimazole. The peaks obtained in the present study were symmetric, good and no interference was observed between the peaks.

Table 1: Retention time of Clotrimazole and Ibuprofen (IS)

S. No	Method	Retention time of Drugs (min)	
		Clotrimazole	Internal Standard (Ibuprofen)
1	HPLC	8.202	5.264
2		8.356	5.272
3		8.242	5.279
4		8.254	5.284
5		8.292	5.296

The method developed was advantageous than the reported methods by its lesser precision values and increased accuracy values.

Chromatographic separation of Clotrimazole in biological fluid:

The chromatogram of the blank plasma was recorded at the fixed chromatographic conditions and shown in figure (**Fig 6)**.

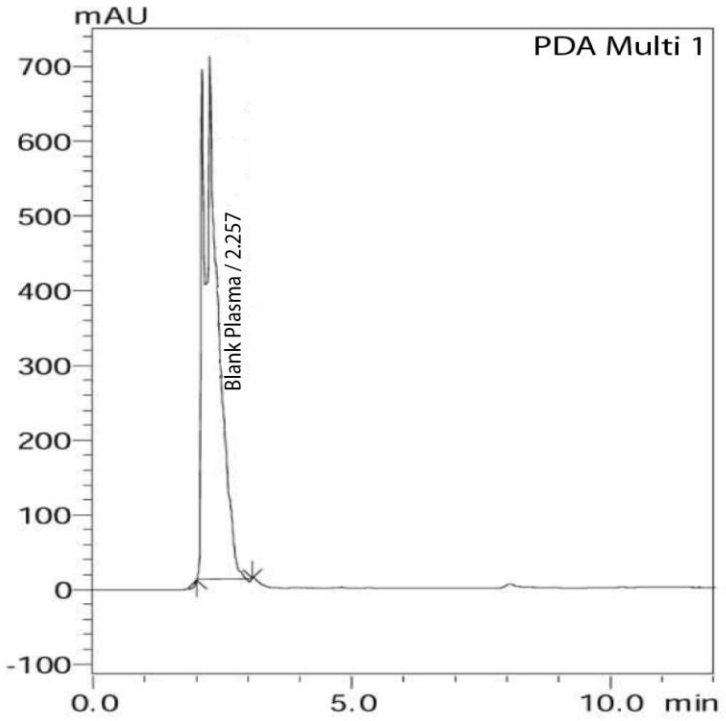


Fig 6 - Chromatogram of blank plasma

Various eluting solvents were used for extraction of Clotrimazole in human plasma. But, out of all eluting solvents acetonitrile-methanol mixture was proved to be good because of its maximum percentage recovery. The percentage recoveries were calculated and shown in table **(Table 2).** The retention time for Clotrimazole and internal standard (Ibuprofen) were 8.3 and 5.2 minutes respectively as shown in **(Fig 5)**. The peaks were symmetric with straight baseline.

Table 2: Recovery study of Clotrimazole

S.No	Conc. of drug added (ng/ml)	Amt. of drug recovered from plasma (ng/ml)			% Recovery		
		ACN	ACN and methanol mixture	Ethyl acetate	ACN	ACN and methanol mixture	Ethyl acetate
I	50	43.2	46.4	48.4	84.2	91.8	86.4
II	100	84.5	88.6	95.2	82.8	92.6	84.2
III	150	124.1	132.4	147.6	83.5	89.9	87.3

*The mean of six value

METHOD VALIDATION

a) Accuracy and precision:

At two –levels these accuracy and precision studies were conducted i.e. intra-day and inter-day. In this the present developed method, shown the good accuracy and precision. Accuracy ranges from 99.99% to 100.4% with the precision 4.10% to 5.84% in intra-day method. In inter-day method the accuracy ranges from 99.7% to 101.07% with the precision 6.65% to 7.88%. Finally the data obtained here, was found to be within limits as per ICH guidelines and method was accurate.

Intra-day studies: In this plasma concentration 50-150 ng/ml were injected six times and mean peak area was calculated separately for each concentration and from that accuracy and precision percentage RSD values were calculated and shown in table **(Table 3)**

Table 3: Accuracy and precision studies of Clotrimazole (Intraday)

Sl.no	Conc. of drug (ng/ml)	Mean peak area	Accuracy (%)	RSD (%)
1	50	14927	99.99	4.10
2	100	32163	101.2	5.84
3	150	48063	100.4	4.45

* The mean of six value

Inter-day studies: In this the plasma concentrations of 50-150 ng/ml were injected into HPLC six times in three different days and mean peak areas were calculated and from that accuracy and precision percentage RSD were calculated and shown in table **(Table 4).** The percentage relative standard deviation of precision for Clotrimazole was less than 15% for the bioanalytical study. The results obtained were within limits.

Acceptance criteria: The percentage RSD value should be less than 15% for bioanalytical study.

Table 4: Accuracy and precision studies of Clotrimazole (Interday)

Sl.no	Conc. of drug (ng/ml)	Mean peak area	Accuracy (%)	RSD (%)
1	50	14906	99.3	4.58
2	100	32158	101.8	7.65
3	150	48028	100.3	6.29

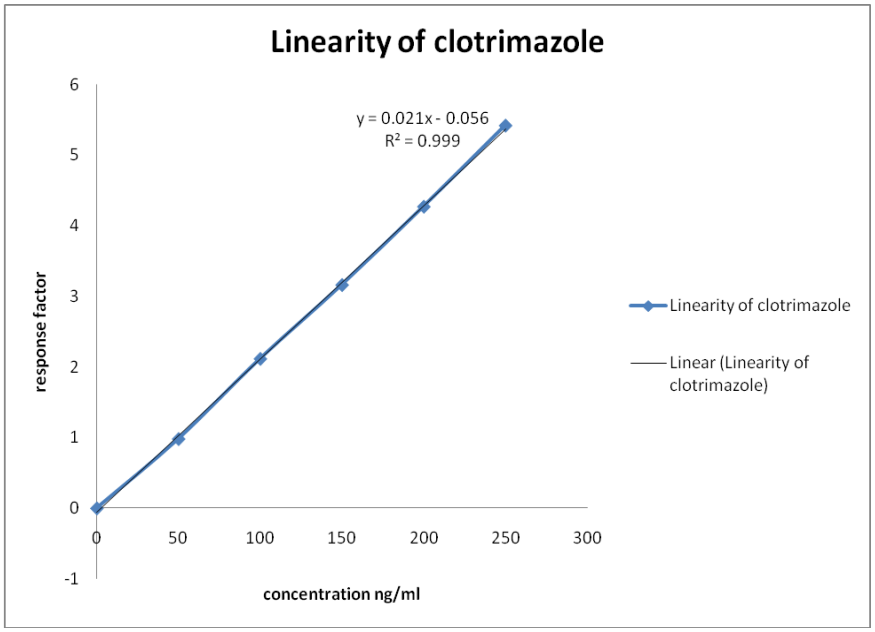
* The mean of six value

b) Linearity and range:

This method proved to be linear between50-250 ng/ml of Clotrimazole in human plasma, with a typical calibration curve of correlation equation y = 0.021x-0.056, correlation coefficient > 0.999 shown in table **(Table 5)**

Table 5: Linearity range of Clotrimazole

Concentration (ng/ml)	Peak area of drug	Peak area of IS	Response factor
0	0	**15186**	0
50	14927		0.983
100	32163		2.118
150	48063		3.165
200	64904		4.274
250	82353		5.423



Calibration curve for Clotrimazole

The chromatograms of the plasma calibration standards with concentrations 50, 100, 150, 200 and 250 ng/ml were recorded and shown in figures **(Fig 7,8,9,10 and 11)** and their peak areas of both drug and internal standard were noted. The calibration curve for Clotrimazole was plotted as peak response Vs concentration of the Clotrimazole calibration standards in plasma **.** As we were using internal standard peak response was calculated for calibration curve. Peak response is the ratio of internal standard peak area to drug peak area. The correlation coefficient of Clotrimazole shown was 0.999 which was within limits. This calibration curve plotted was linear and showed that the method had adequate sensitivity to the concentration (50 ng/ml-250 ng/ml) of the drug. Finally the data obtained, in this was within limits. Coefficient of correlation of Clotrimazole was found to be less than 0.999.

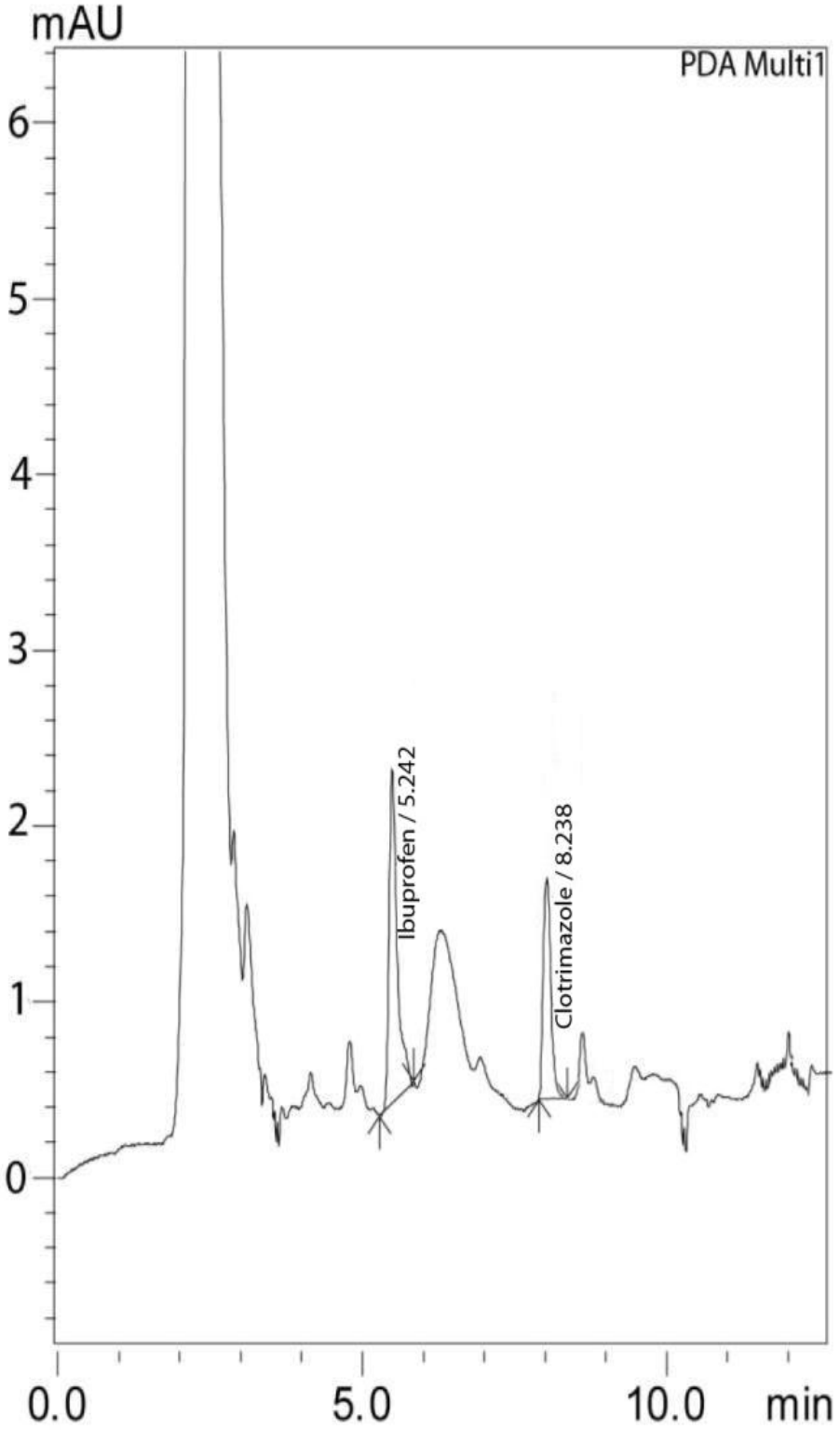


Fig 7 - Chromatogram of IS(100ng/ml) and Clotrimazole (50ng/ml) in human plasma

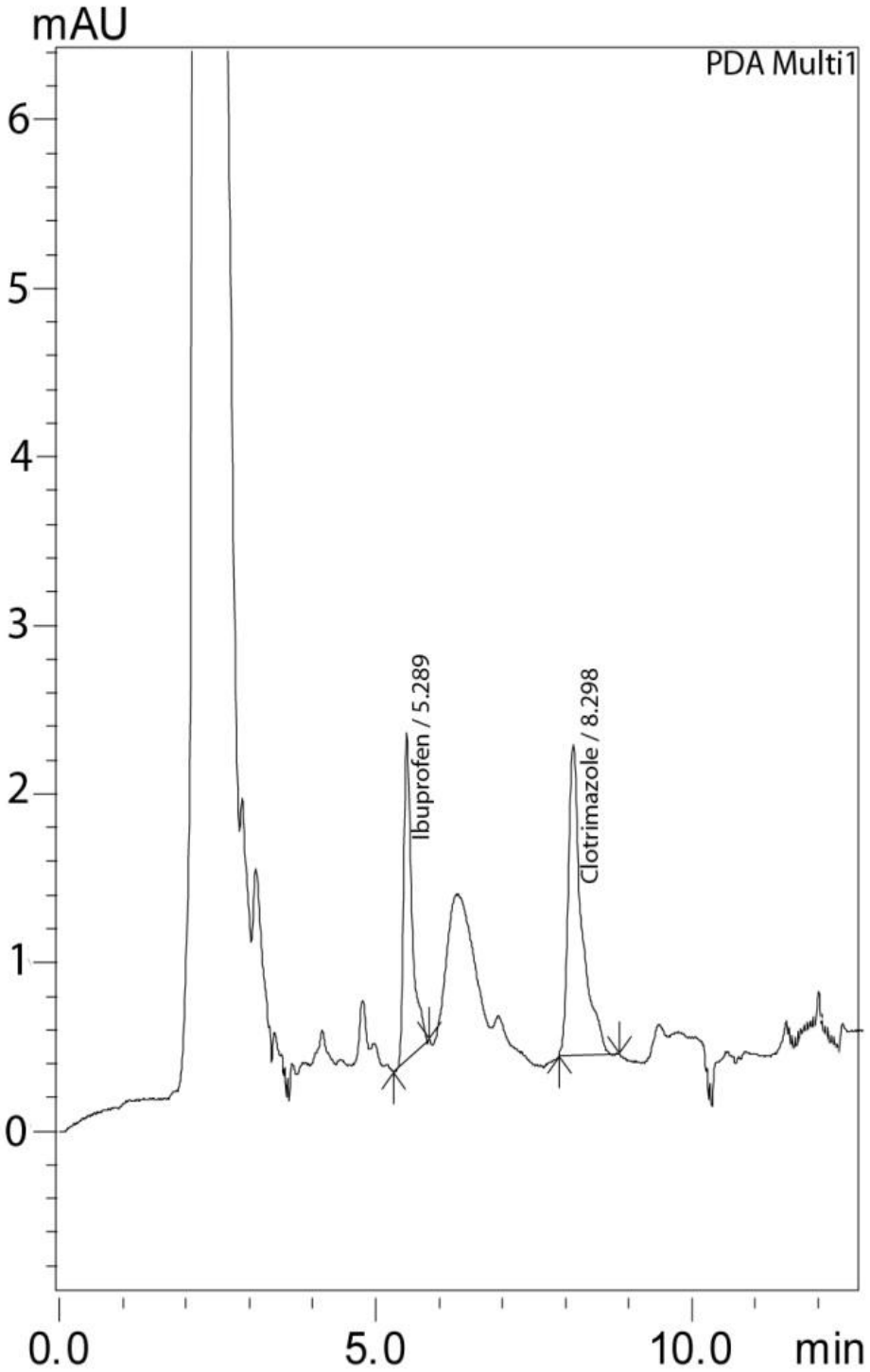


Fig 8 - Chromatogram of Ibuprofen (100ng/ml) and Clotrimazole (100ng/ml) in human plasma

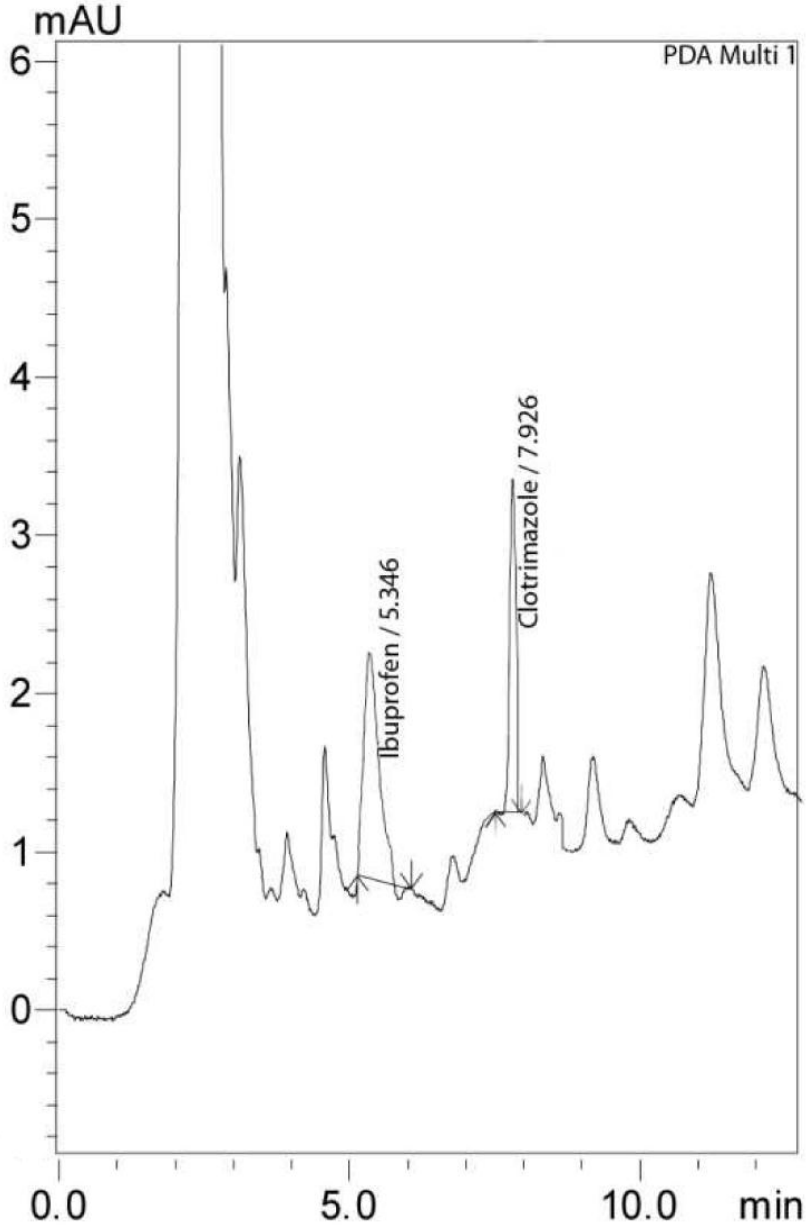


Fig 9 - Chromatogram of Ibuprofen (100ng/ml) and Clotrimazole (150ng/ml) in human plasma

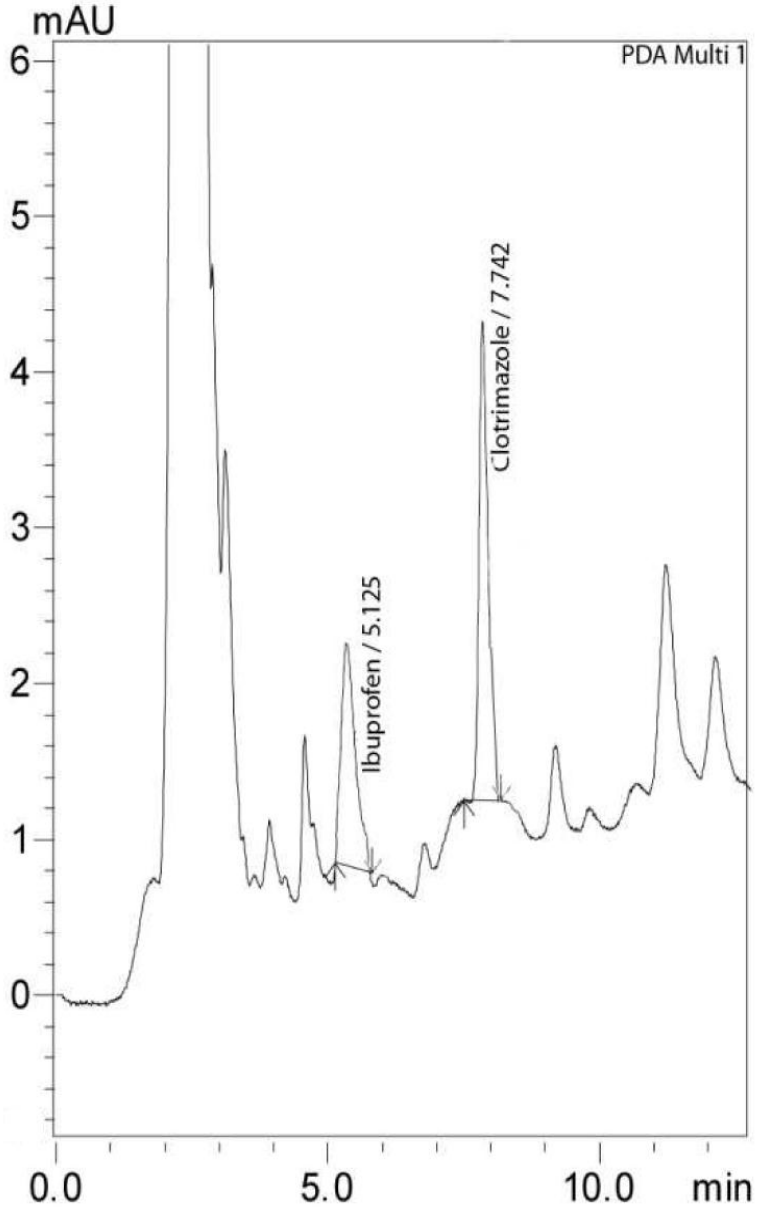


Fig 10- Chromatogram of Ibuprofen (100ng/ml) and Clotrimazole (200ng/ml) in human plasma

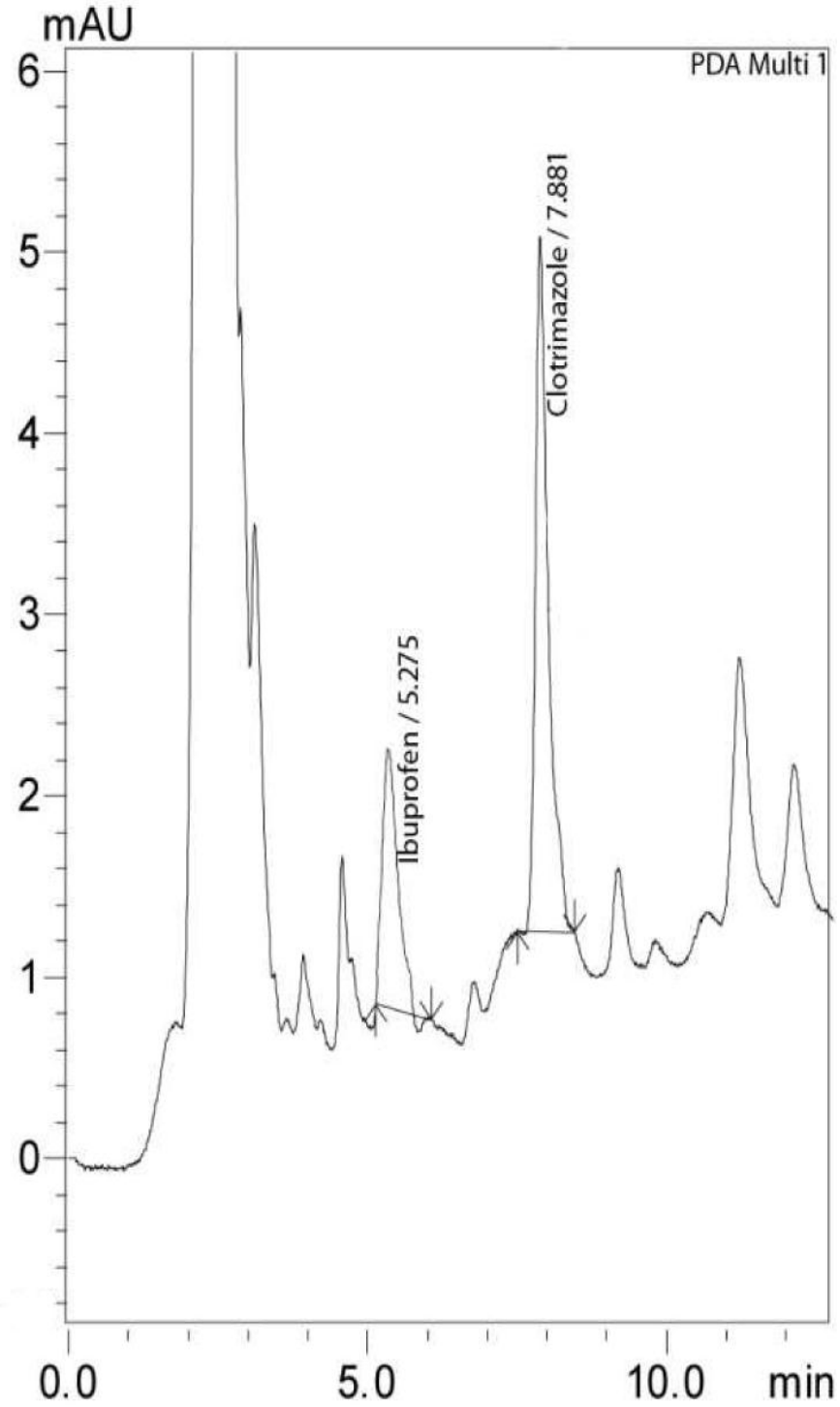


Fig 11 - Chromatogram of Ibuprofen (100ng/ml) and Clotrimazole (250ng/ml) in human plasma

c) Lower Limit of Quantification and Limit of Detection:

The LOD is the smallest concentration of the analyte which shows a measurable response .The LOQ is the smallest concentration of the analyte, which shows response that can be accurately quantified LOD = 3.3 × D/S and LOQ = 10 × D/S, where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve. This signal to noise ratio were performed by comparing measured signal of known low concentration of drug with those of blank plasma sample.

The Lower limit of quantification (LOQ) and limit of detection (LOD) for Clotrimazole were separately determined and reported, based on the calibration curve for spiked plasma solutions was found to be 23.90 ng/ml and 7.8 ng/ml Srespectively.

d) Recovery from plasma:

A recovery study for Clotrimazole in plasma using acetonitrile-methanol mixture was shown in table **(Table 6)**. With concentrations 50 ng/ml, 100 ng/ml, 150 ng/ml of Clotrimazole recovery was calculated and showed 98.2%, 100.2%, 101.4% relative recoveries and percentage RSD as4.6%, 5.3% and 6.9% respectively.

Acceptance criteria: For an assay method, mean recovery should be 85-105% ± 2%.

Table 6: Recovery studies of Clotrimazole

Levels	Conc. of drug added (ng/ml)	Amt of drug recovered in plasma sample (ng /ml)	Percentage recovery (%)	% RSD
I	50	49.5	100.2	4.6
II	100	98.9	98.2	6.9
III	150	149.1	101.4	5.3

* The mean of six value

.

e) Ruggedness:

It expresses the precision within laboratories variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the plasma standard 6 times in two different days with different analyst and the standard deviation were analyzed by a different chemist and same instruments on a different day had been performed the reports were shown in table **(Table 7)**.

The deviation among the results obtained by two chemists on a different day was well within the limits. Hence the method was rugged.

Acceptance criteria: The percentage RSD should be less than 15%.

Table 7: Ruggedness studies for Clotrimazole

Drug	Concentration (ng/ml)	Mean peak area	%RSD
	Day I analyst – I		
Clotrimazole	50	14910	4.267.
	Day II analyst – II		
Clotrimazole	50	14908	4.148

* The mean of six value

.

f) Specificity:

For specificity the peak purity studies were done. Here for Clotrimazole the peak purity index was 000 and the peak properties like peak profile were good for both standard and the sample. The peak purity and peak profiles for Clotrimazole standard and sample were shown in figures **(Fig 12, 13, 14, and 15)** respectively. By the data obtained in this, the present method developed was specific as values were within limits.

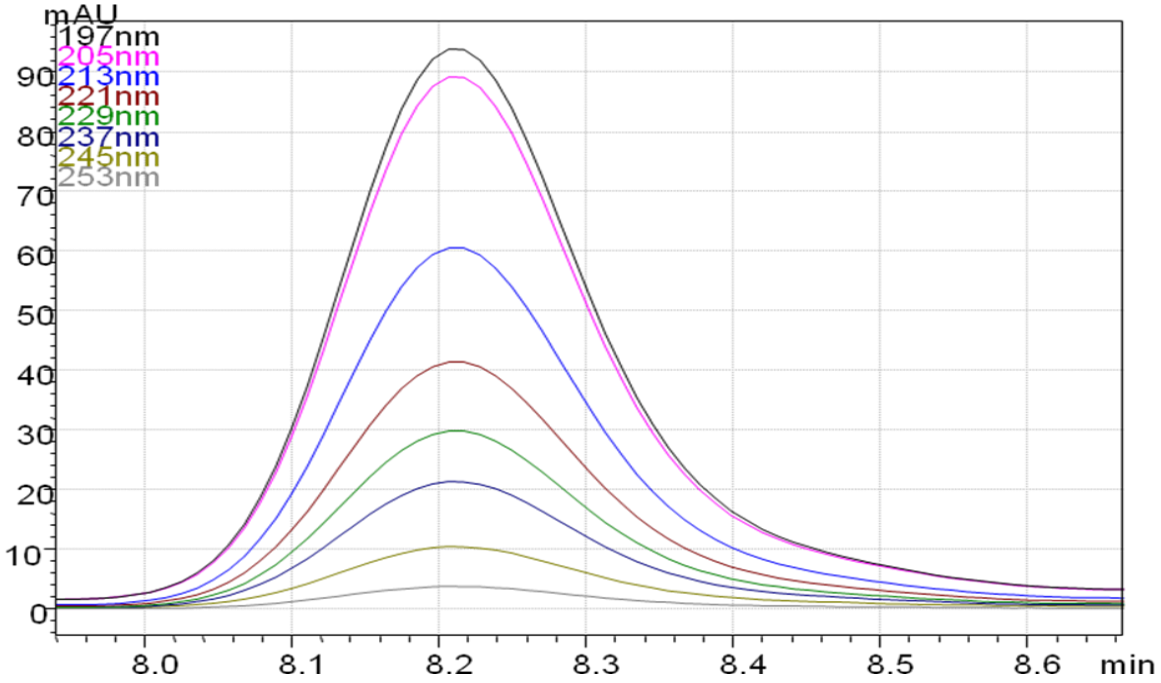


Fig 12 - Peak profile of standard Clotrimazole

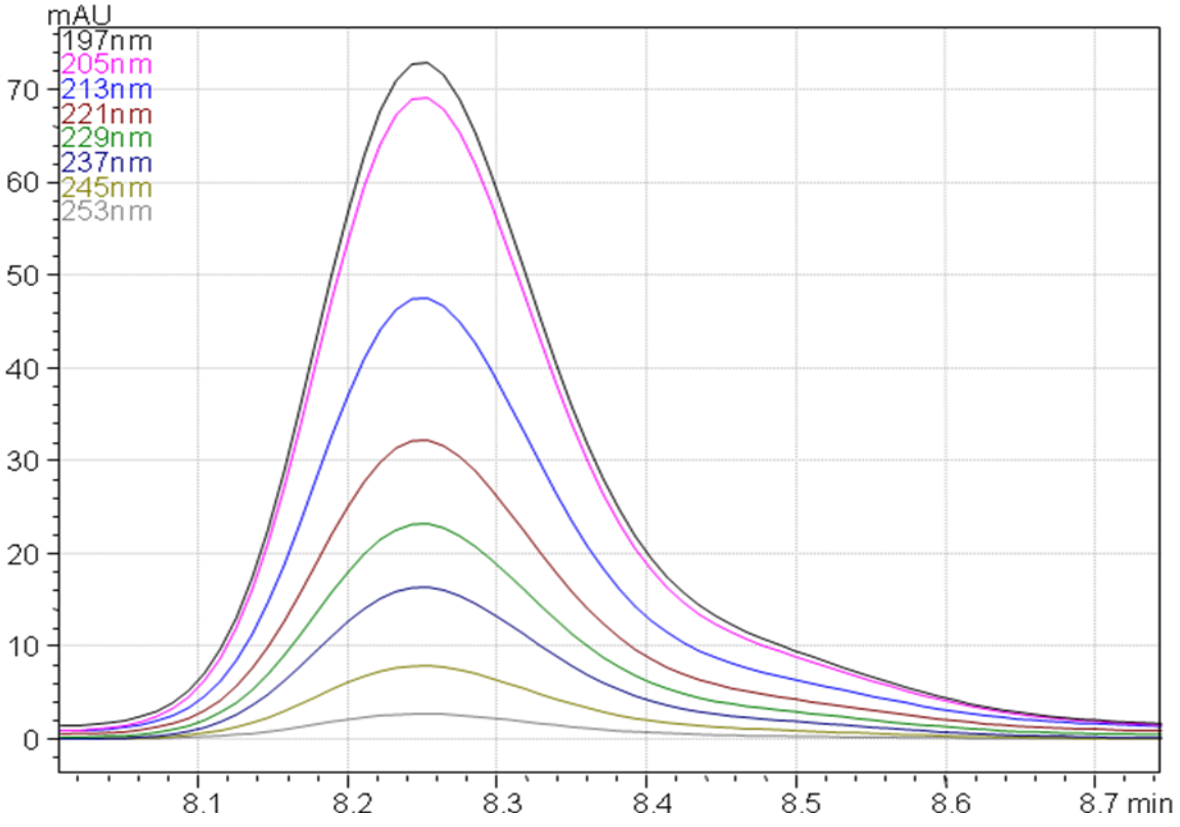


Fig 13 - Peak profile of Clotrimazole in human plasma

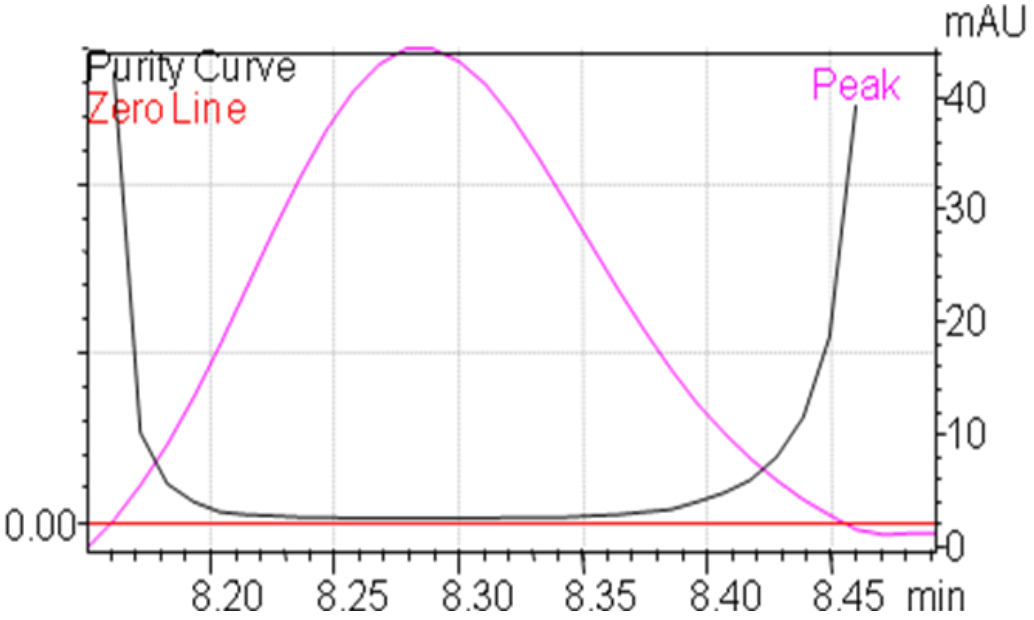


Fig 14 - Peak purity of standard Clotrimazole

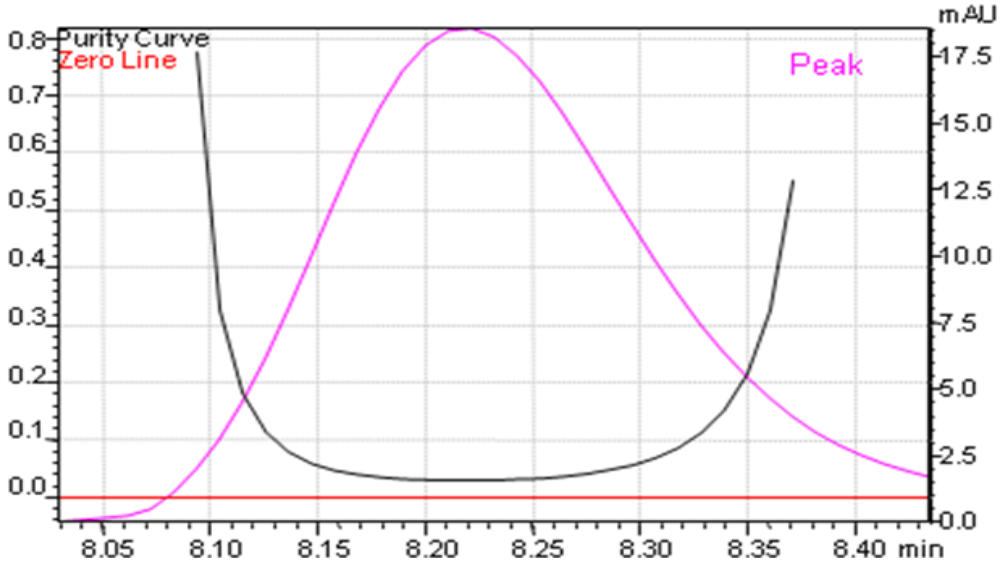


Fig 15 - Peak purity of Clotrimazole in human plasma

g) System suitability:

These parameters were shown to be within specified limits. Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, HETP, tailing factor, LOQ and LOD are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table **(Table 8).** These parameters were shown to be within specified limits.

Table 8: System suitability studies

Sl.No	Parameters	Clotrimazole
1.	Theoretical Plate	6662
2.	Tailing Factor	1.1
3.	HETP	22.51
4.	LOQ	23.90 ng/ml
5.	LOD	7.8 ng/ml
6.	Resolution	5.8
7.	K	2.83

8. SUMMARY AND CONCLUSION

- A bioanalytical method was developed for the estimation of Clotrimazole by HPLC method.
- The method was validated for its transferability to other user or other laboratory.
- The HPLC method was developed by using 0.5%TEA in water (pH 3 adjusted with Orthophosphoric acid) and Acetonitrile in meticulous ratio.
- The peaks obtained for the drugs of interest by the present method are well resolved from each other without any interference and from the plasma endogenous proteins.
- The peaks are symmetrical with acceptable tailing factor.
- The retention time of Clotrimazole was within the limit.
- The results of linearity, intraday and interday precision study and capability of the extraction method were within the limits of bioanalytical method development.
- The method was linear with a correlation coefficient of acceptable agreement, which is suitable for the estimation of Clotrimazole in human plasma and other biological fluids.
- The method demonstrated relative recoveries with acceptable relative standard deviation.
- The limit of quantification (LOQ) and limit of detection (LOD) for Clotrimazole was found to be nanograms lesser than unity.
- Hence the developed method is sensitive for the estimation of Clotrimazole in trace amounts.
- Peak purity studies, with peak purity index values closer to unity reveals that the method developed was specific for the estimation of Clotrimazole in blood and other biological fluids.
- It can be concluded that the developed RP-HPLC method in human plasma was found to be very simple, reliable and selective for providing satisfactory accuracy and precision.
- The methods are suitable for routine quantitative analysis in pharmaceutical dosage forms. Hence this developed method can be used further in
    - Bioequivalence and bioavailability studies of clotrimazole.
    - Pharmacokinetic and bio-equivalence study centers

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ABSTRACT

A sensitive and selective HPLC method for the estimation of Clotrimazole in human plasma was developed and validated. Sample preparation was carried out using protein precipitation method. Separation was done on a Enable C18 reverse phase column with a mobile phase of 0.5% TEA in water (pH 3 adjusted with o-phosphoric acid) and acetonitrile in the ratio of 20:80%v/v and detection at 215nm. The retention time of 5.2 and 8.2mins for ibuprofen(IS) and clotrimazole is highly advantageous. The standard curve is linear (r>0.999) over the concentration range of 50-250ng/ml. Validated analytical method was based on the parameters: selectivity, accuracy, precision, linearity, LOD, LOQ and the suitability of the system. Validation analysis showed selectivity test Rs.1.5, based on the area ratio of peak height and a segment of the chromatogram obtained LOD Value 7.8 ng/ml. LOQ value of 23.90ng/ml, percent accuracy from 99.5-100.3. For interday studies and 99.9-101.4 for intraday studies and precision 4.58-6.5 for interday studies and 4.10-5.84 for intraday studies. System suitability test results on the retention time, area ratio and peak ratios of peak chromatogram shows the CV(%)<10%. Thus it is concluded that the analytical methods used have validity in accordance with requirements.

Keywords: RP-HPLC, Clotrimazole. Protein precipitation method, Human plasma